

RECEIVED #7

10 JAN 2007

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FACSIMILE COVER SHEET
PLEASE DELIVER IMMEDIATELY!!!!

Our Ref.: 620-359

Your Ref.: Appln No. 10/523,593

Date: January 10, 2007

To: Ms Cynthia M. Kratz

Firm: USPTO - Office of PCT Legal Administration-

Facsimile No.: Facsimile No.: 571-273-0459 (Examiner's Tel. No.: 5712723286)

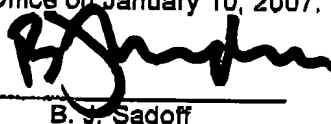
From: B. J. Sadoff

Number of Pages (including cover sheet): 114

(IF YOU DO NOT RECEIVE ALL OF THE PAGES OR ENCOUNTER DIFFICULTIES IN TRANSMISSION,
PLEASE CONTACT US IMMEDIATELY AT (703-816-4000).

CERTIFICATION OF FACSIMILE TRANSMISSION

I hereby certify that this paper and any noted attachments are being facsimile transmitted to the Patent and Trademark Office on January 10, 2007.


B. J. Sadoff

ATTACHMENT/S: Rule 182 Petition; (1) USPTO date-stamped post card receipt from submission of January 3, 2007; (2) USPTO date-stamped post card receipt from submission of November 2, 2006; (3) copy of Information Disclosure Statement and PTO 1449 Form filed January 3, 2007 (dated January 2, 2007); (4) copy of WO 2004/020639 filed January 3, 2007; (5) copy of Christiansen et al, Journal of Virology, May 2000, 74, No. 10, pp 4672-4678, filed January 3, 2007; (6) copy of Liby et al, Blood, November 15, 1997, Vol. 90, No. 10, pp 3978-3983, filed January 3, 2007; (7) copy of International Search Report dated July 18, 2005, issued in connection with PCT/IB2004/002717, filed January 3, 2007; (8) copy of cover letter filed November 2, 2006; (9) copy of Renewed Petition Under 37 CFR 1.47(a) filed November 2, 2006; (10) copy of Declaration (6 pages) filed as a part of Renewed Petition on November 2, 2006; and (11) copy of Supplemental Application Data Sheet filed November 2, 2006.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE			
In re Patent Application of			
ANDREOLETTI, ET AL.		Atty. Ref.:	620-359
Serial No.	10/523,593	Group:	Unassigned
Filed:	February 4, 2005	Examiner:	Unassigned
For:	HETEROPOLYMERIC COMPOUND COMPRISING A SCAFFOLD, AN ADJUVANT AND AN ANTIGEN, AND ITS USE		

CONFIDENTIALITY NOTE

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1159721

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

ANDREOLETTI, ET AL.

Atty. Ref.: 620-359

Serial No. 10/523,593

Group: Unassigned

Filed: February 4, 2005

Examiner: Unassigned

For: HETEROPOLYMERIC COMPOUND COMPRISING A SCAFFOLD, AN ADJUVANT
AND AN ANTIGEN, AND ITS USE

* * * * *

January 10, 2007

Mail Stop PCT

Commissioner for Patents
Office of PCT Legal Administration
P.O. Box 1450
Alexandria, VA 22313-1450

via fax: 571-273-0459

**Attn: Office of PCT Legal Administration
Ms Cynthia M. Kratz**

Sir:

RULE 182 PETITION

Responsive to the telephone message from Ms Kratz received by the undersigned on January 10, 2007, attached is a copy of the following:

- (1) USPTO date-stamped post card receipt from submission of January 3, 2007;
- (2) USPTO date-stamped post card receipt from submission of November 2, 2006;
- (3) copy of Information Disclosure Statement and PTO 1449 Form filed January 3, 2007 (dated January 2, 2007);
- (4) copy of WO 2004/020639 filed January 3, 2007;
- (5) copy of Christiansen et al, Journal of Virology, May 2000, 74, No. 10, pp 4672-4678, filed January 3, 2007;

ANDREOLETTI, ET AL.
Serial No. 10/523,593
January 10, 2007
Rule 182 petition

(6) copy of Libyh et al, Blood, November 15, 1997, Vol. 90, No. 10, pp 3978-3983, filed January 3, 2007;

(7) copy of International Search Report dated July 18, 2005, issued in connection with PCT/IB2004/002717, filed January 3, 2007;

(8) copy of cover letter filed November 2, 2006;

(9) copy of Renewed Petition Under 37 CFR 1.47(a) filed November 2, 2006;

(10) copy of Declaration (6 pages) filed as a part of Renewed Petition on November 2, 2006; and

(11) copy of Supplemental Application Data Sheet filed November 2, 2006.

The attached is being resubmitted as a review of the PTO IFW indicates that the same has not been scanned and entered and Ms Kratz confirmed in the telephone message of January 10, 2007 that the attached does not appear to have been received by the Patent Office.

The attached post card receipts are submitted as evidence of receipt of the attached papers on the indicated dates.

The present paper is being submitted in the form of a Petition, as suggested by Ms Kratz. No fee is believed to be required for consideration of the present Petition as the originally-filed papers are believed to have been received by the Patent Office however the Office is authorized by the attached cover letter to charge the undersigned's Deposit Account No. 14-1140 for any fee required to consider the present Petition.

Grant of the present Petition and favorable consideration of the attached Renewed Petition are requested along with a Notification of Acceptance or other paper

ANDREOLETTI, ET AL.Serial No. **10/523,593**

January 10, 2007

Rule 182 petition

notifying the undersigned that the present application has been forwarded to OIPE for further docketing and action as a 371 U.S. National Phase application.

The Office is requested to contact the undersigned in the event anything further is required.

Respectfully submitted,

NIXON & VANDERHYE P.C.By: /B. J. Sadoff/

B. J. Sadoff

Reg. No. 36,663

BJS:

901 North Glebe Road, 11th Floor

Arlington, VA 22203-1808

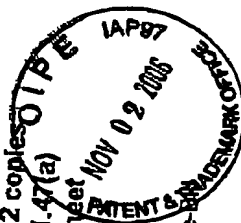
Telephone: (703) 816-4000

Facsimile: (703) 816-4100

COPY

Serial No.: 10/523,593
Inventor/s: ANDREOLETTI, ET AL.
Title: HETEROPOLYMERIC COMPOUND COMPRISING
A SCAFFOLD, AN ADJUVANT AND AN ANTIGEN, AND
ITS USE
C#/##: 620-359
Atty: B. J. Sadoff
Date: 11/2/2006

XX Cover Sheet/Extension of Time - 2 copies
XX Renewed Petition Under 37 CFR 1.47(a)
XX Supplemental Application Data Sheet



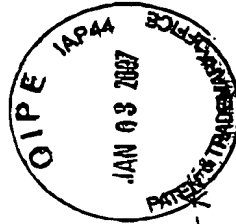
\$ Fee (Check) - Pre-Bill
\$ 120.00 Fee (Check) - Non Pre-Bill
\$ 120.00 Total Fee Enclosed

Others: Declaration (6 pages)

COPY

C#/#: 620-359
 Atty: B. J. Sadoff
 Date: 1/3/2007
 Serial No.: 10/523,593
 Inventor/s: ANDREOLETTI, ET AL.
 Title: HETEROPOLYMERIC COMPOUND COMPRISING
 A SCAFFOLD, AN ADJUVANT AND AN ANTIGEN, AND
 ITS USE

XX IDS, PTO-1449 Form & non-U.S. patent references cited
 ITS USE



\$ Fee (Check) - Pre-Bill
 \$ Fee (Check) - Non Pre-Bill
 \$ Total Fee Enclosed

Others: *International Search Report*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

Atty 620-359
Dkt.

C# M#

ANDREOLETTI, ET AL.

TC/A.U. Unassigned

Serial No. 10/523,593

Examiner: Unassigned

Filed: February 4, 2005

Date: November 2, 2006

Title: HETEROPOLYMERIC COMPOUND COMPRISING A SCAFFOLD, AN ADJUVANT
AND AN ANTIGEN, AND ITS USE**Mail Stop PCT**Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450**Attn: Office of PCT Legal Administration**

Sir:

RENEWED PETITION UNDER 37 CFR 1.47(a)

This is a response/amendment/letter in the above-identified application and includes an attachment which is hereby incorporated by reference and the signature below serves as the signature to the attachment in the absence of any other signature thereon.

☐ **Correspondence Address Indication Form Attached.****Fees are attached as calculated below:**

Total effective claims after amendment	0	minus highest number	
previously paid for	20 (at least 20) =	0 x \$50.00	\$0.00 (1202)/\$0.00 (2202) \$

Independent claims after amendment	0	minus highest number	
previously paid for	3 (at least 3) =	0 x \$200.00	\$0.00 (1201)/\$0.00 (2201) \$

If proper multiple dependent claims now added for first time, (ignore improper); add
\$360.00 (1203)/\$180.00 (2203) \$

Petition is hereby made to extend the current due date so as to cover the filing date of this
paper and attachment(s)

One Month Extension	\$120.00 (1251)/\$60.00 (2251)	
Two Month Extensions	\$450.00 (1252)/\$225.00 (2252)	
Three Month Extensions	\$1020.00 (1253)/\$510.00 (2253)	
Four Month Extensions	\$1590.00 (1254)/\$795.00 (2254)	
Five Month Extensions	\$2160.00 (1255)/\$1080.00 (2255)	\$ 120.00
	\$130.00 (1814)/ \$65.00 (2814)	\$

Terminal disclaimer enclosed, add

☐ Applicant claims "small entity" status. ☐ Statement filed herewith

Rule 56 Information Disclosure Statement Filing Fee	\$180.00 (1806)	\$	0.00
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Assignment Recording Fee	\$40.00 (8021)	\$	0.00
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Other:		\$	0.00
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TOTAL FEE ENCLOSED \$ 120.00

The Commissioner is hereby authorized to charge any deficiency, or credit any overpayment, in the fee(s) filed, or asserted to be filed, or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Account No. 14-1140. A duplicate copy of this sheet is attached.

901 North Glebe Road, 11th Floor
Arlington, Virginia 22203-1808
Telephone: (703) 816-4000
Facsimile: (703) 816-4100
3JS:ppNIXON & VANDERHYE P.C.
By Atty: B. J. Sadoff, Reg. No. 36,663Signature: 

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

ANDREOLETTI, ET AL.

Atty. Ref.: 620-359

Serial No. 10/523,593

Group: Unassigned

Filed: February 4, 2005

Examiner: Unassigned

For: HETEROPOLYMERIC COMPOUND COMPRISING A SCAFFOLD, AN ADJUVANT
AND AN ANTIGEN, AND ITS USE

* * * * *

November 2, 2006

Mail Stop PCT

Commissioner for Patents

Office of PCT Legal Administration

P.O. Box 1450

Alexandria, VA 22313-1450

Attn: Office of PCT Legal Administration

Sir:

RENEWED PETITION UNDER 37 CFR 1.47(a)

Receipt of the DECISION dated August 3, 2006, is hereby acknowledged.

Attached is a completely executed Inventors Declaration (3 copies, 6 pages total) which have been further executed by Pierre Andreoletti, Michel Julien and Emmanuel Risse.

Also attached is a Supplemental Application Data Sheet which includes the new addresses of Pierre Andreoletti, Michel Julien and Emmanuel Risse.

The Rule 47 Petition and Statement in support thereof dated February 27, 2006 is believed to be moot in view of the attached. The Office is requested to forward the application to OIPE, or other appropriate Office, for docketing and assignment to an examiner for examination on the merits. Alternatively, the Office is requested to advise

ANDREOLETTI, ET AL.

Serial No. 10/523,593

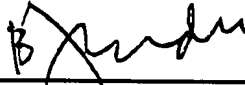
November 2, 2006

the undersigned in the event anything further is required to complete the filing of this
U.S. national phase of PCT/EP2003/008926.

Respectfully submitted,

NIXON & VANDERHYE P.C.

By: _____


B. J. Sadoff
Reg. No. 36,663

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Arlington, VA 22203-1808
Telephone: (703) 816-4000
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

ANDREOLETTI, ET AL.

Atty. Ref.: 620-359

Serial No. 10/523,593

Group: 1645

Filed: February 4, 2005

Examiner: Unassigned

For: HETEROPOLYMERIC COMPOUND COMPRISING A
SCAFFOLD, AN ADJUVANT AND AN ANTIGEN, AND ITS
USE

* * * * *

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

January 2, 2007

Sir:

INFORMATION DISCLOSURE STATEMENT

- ☒ 1. **PTO-1449 Pursuant to 37 CFR 1.97(b)**
[within 3 months of filing or prior to 1st Office Action on the merits]
N/C
- ☐ 2.(a) **Statement Pursuant to 37 CFR 1.97(c)**
[before Final Office Action or Allowance (requires Rule 97(e)
Statement or Rule 17(p) fee)]
N/C
- ☐ 2.(b) **Fee Payment Pursuant to 37 CFR 1.97(c)**
[before Final Office Action or Allowance (requires Rule 97(e)
Statement or Rule 17(p) fee)]
\$180.00
- ☐ 3. **Pursuant to 37 CFR 1.97(d)**
[after Final Office Action or Allowance (requires Rule 97(e)
Statement and Rule 17(p) fee), but before final fee payment]
\$180.00

1155145

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ANDREOLETTI, ET AL.
Serial No. 10/523,59

The following are submitted in the above-identified application in compliance with 37 C.F.R. §§ 1.97 and 1.98:

- ☒ 4. A list of documents on Form PTO-1449 together with copies of each identified document and a translation or a concise explanation of each non-English language document (such as a Search Report) is enclosed herewith.

This paper is submitted in accordance with:

- ☒ 5. 37 CFR 1.97(b): [within 3 months of filing or prior to 1st Office Action]
- ☐ 6. 37 CFR 1.97(c): [before Final Office Action or Allowance, whichever is earlier]; and
- ☐ a) The required Statement made in item 8 below; or
- ☐ b) The \$180.00 fee specified in 37 CFR §1.17(p) for submission of this Information Disclosure Statement is authorized in item 9 below.
- ☐ 7. 37 CFR §1.97(d): [after Final Office Action or Allowance (requires Rule 97(e) Statement and Rule 17(p) fee), but before final fee payment]; and
- ☐ a) The fee (\$180.00) required by 37 CFR §1.17(p) is submitted herewith; and
- ☐ b) The required Statement is stated in item 8 below.
- ☐ 8. Statement under 37 CFR 1.97(e)
- ☐ a) The undersigned attorney of record hereby certifies under 37 C.F.R. §1.97(e) that each item of information contained in this Information Disclosure Statement was first cited in a communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Information Disclosure Statement (each item contained in this IDS was the first citation of that item by a foreign patent office in a counterpart foreign application which occurred no more than three months prior to the filing of this IDS); or
- ☐ b) No item of information contained in this Information Disclosure Statement was cited in a communication from a foreign patent office in a counterpart foreign application, and, to the knowledge of the person signing this Statement, after making reasonable inquiry, no item of information contained in this Statement was known to any individual designated in 37 CFR §1.56(c) more than three months prior to the filing of this Information Disclosure Statement.

ANDREOLETTI, ET AL.
Serial No. 10/523,593

- ☒ 9. Please charge all deficiency fees associated with the submission of this Information Disclosure Statement and any other fees applicable to this application to Deposit Account No. 14-1140. An original and one (1) copy of this document are enclosed.

Respectfully submitted,
NIXON & VANDERHYE P.C.

By: _____


B. J. Sadoff

Reg. No. 36,663

901 North Glebe Road, 11th Floor
Arlington, VA 22203-1808
Telephone: (703) 816-4000
Facsimile: (703) 816-4100

Sheet 1 of 1

**INFORMATION DISCLOSURE
CITATION**

ATTY. DOCKET NO.

620-359

APPLICANT

SERIAL NO.

10/523,593

(Use several sheets if necessary)

ANDREOLETTI, ET AL.

FILING DATE

February 4, 2005

GROUP

Unassigned

U.S. PATENT DOCUMENTS

[illegible]

FOREIGN PATENT DOCUMENTS

[illegible]

OTHER DOCUMENTS (including Author, Title, Date, Pertinent pages, etc.)

	Christiansen et al, Journal of Virology, May 2000, 74, No. 10, pp 4672-4678
	Libyh et al, Blood, November 15, 1997, Vol. 90, No. 10, pp 3978-3983
	International Search Report dated July 18, 2005, issued in connection with PCT/IB2004/002717

Examiner

Date Considered

Examiner: Initial if reference considered, whether or not citation is in conformance with MPEP 609; Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to application.

Form PTO-FB-A820 (Also PTO-1448)

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1155145

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
11 March 2004 (11.03.2004)

PCT

(10) International Publication Number
WO 2004/020639 A2

(51) International Patent Classification⁷: C12N 15/62

(21) International Application Number:
PCT/EP2003/008928

(22) International Filing Date: 12 August 2003 (12.08.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02292043.3 14 August 2002 (14.08.2002) EP

(71) Applicant (for all designated States except US): AVIDIS SA [FR/FR]; Biopole Clermont-Limagne, F-63360 Saint-Beauzire (FR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): GARNIER, Laurence [FR/FR]; Avidis SA, Biopole Clermont-Limagne, F-63360 Saint-Beauzire (FR). HILL, Fergal [IE/FR]; Avidis SA, Biopole Clermont-Limagne, F-63360 Saint-Beauzire (FR). JULIEN, Michel [FR/FR]; Avidis SA, Biopole Clermont-Limagne, F-63360 Saint-Beauzire (FR).

(74) Agents: BRASNETT, Adrian, H. et al.; Mewburn Ellis, York House, 23 Kingsway, London, Greater London WC2B 6HP (GB).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PRODUCTION OF MULTIMERIC FUSION PROTEINS USING A C4BP SCAFFOLD

(57) Abstract: The present invention provides a method for obtaining a recombinant fusion protein comprising a scaffold of a C-terminal core protein of C4bp alpha chain, said recombinant fusion protein being capable of forming multimers in soluble form in a prokaryotic host cell, the method including the steps of (i) providing a prokaryotic host cell carrying a nucleic acid encoding said recombinant protein operably linked to a promoter functional in said prokaryotic cell; (ii) culturing the host cell under conditions wherein said recombinant protein is expressed; and (iii) recovering the recombinant protein wherein said protein is recovered in multimeric form without performing a scaffold refolding step.

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WO 2004/020639 A2

PRODUCTION OF MULTIMERIC FUSION PROTEINS USING A C4BP SCAFFOLDIntroduction.

5 This invention relates to methods for producing high yields of fusion proteins and polypeptides comprising a C4bp domain in prokaryotic cells.

Background of the Invention.

10 The advent of recombinant DNA technology has provided the possibility of large scale production of biologically active proteins for therapeutic use. There are now many recombinant DNA produced products in the clinic or under development, including large proteins such as erythropoietin, small peptides, and antibody fragments.

15 It is known in the art that a difficulty with proteins is one of half life. Many proteins and peptides have a short half-life *in vivo*, reducing their usefulness. It has been found that multimerisation of protein and peptide molecules is a way
20 of increasing the half-life of these molecules thus allowing them to exert their activity over a longer time scale. Many functional biological molecules have been found to be more potent *in vivo* when in the form of an oligomeric structure. This is due to factors such as binding with avidity rather
25 than affinity, and/or the ability to cross-link molecules (e.g. identical receptor subunits as in the insulin receptor that are activated through dimerisation, or non-identical molecules to form signalling complexes on the cell surface, such as in lymphocytes). These properties of increased half-
30 life and avidity enable lower doses of the protein and peptide

molecules to be used, thereby reducing costs and dose-dependent side-effects.

Different approaches have been proposed for making multimers of recombinant proteins. For example, chemical linkage of proteins to polymers such as polyethylene glycol has been attempted (Katre et al., (1987) Proc. Natl. Acad. Sci. USA 84, 1487). This technique, however, is cumbersome and requires large amounts of purified material. In antibody molecules, modifications of the disulphide-forming possibilities in the hinge, and other regions of the molecules have been attempted in order to modulate the extent to which antibodies will associate with each other. Results however have been inconsistent and unpredictable. Similarly, use of protein A fusions to generate multimeric antibodies may successfully link antibody fragments, but is of limited application in other fields.

A new multimerisation system using the complement 4 binding protein (C4bp) is described in WO 91/11461. Human C4-binding protein (C4bp) is a plasma glycoprotein of high molecular mass (570 kDa) which has a spider like structure made of seven identical alpha-chains and a single beta-chain. The C4bp alpha chain has a C-terminal core region responsible for assembly of the molecule into a multimer. According to the standard model, the cysteine at position +498 of one C4bp monomer forms a disulphide bond with the cysteine at position +510 of another monomer. A minor form comprising only seven alpha-chains has also been found in human plasma. The natural function of this plasma glycoprotein is to inhibit the classical pathway of complement activation.

WO 91/11461 proposes that the ability of the C4bp protein to multimerise can be used to make fusion proteins comprising all or part of C4bp and a biological protein of interest. The fusion protein will form multimers which provides a platform for the protein of interest, in which said protein has an enhanced serum half-life and increased affinity or avidity for its targets. Fusion proteins of C4bp were targeted as the focus of novel delivery and carrier systems for therapeutic products in WO 91/11461.

Most of the alpha-chain of C4bp is composed of eight tandemly arranged domains of approximately 60 amino acids in length known as complement control protein (CCP) repeats. Inclusion of one or more of these domains was preferred in the fusion proteins described in WO 91/11461, but it has since been demonstrated that all CCPs can be deleted (leaving only the C-terminal 57 amino acids) without preventing multimerisation (Libyh M. T. et al., (1997) Blood 90, 3978). This C-terminal region of C4bp is referred to as the C4bp core.

Libyh et al. (1997), describe a protein multimerisation system which is based on the C-terminal part of the alpha chain of C4bp. The C-terminal part of the C4bp lacks biological function, but is responsible for polymerisation of C4bp in the cytoplasm of CHO cells producing C4bp. Libyh et al. were able to induce spontaneous multimerisation of associated antibody fragments to create homomultimers of scFv fragments using the C4bp fragment. The C-terminal portion of C4bp used was placed C-terminal to the scFv sequence, optionally spaced by a MYC tag.

Oudin et al. (2000, Journal of Immunology, 164, 1505) further use the C4bp core multimerising system for forming hetero-

multimeric multi CR1/scFv anti-Rh(D) molecules. The chimera proteins were expressed in a CHO cell line by co-transfection of these cells and by two different vectors (one encoding CR1 and the other encoding ScFv anti Rh-D) and were found to spontaneously multimerise in the cytoplasm of the transfected cells from which they were secreted.

Christiansen et al. (2000, Journal of Virology, 74, 4672) further demonstrate the production of homo-multimeric fusion proteins encompassing the CD46 ectodomain linked to the C4bp core in 293 EBNA cells.

Self-assembling multimeric soluble CD4-C4bp fusion protein have also been demonstrated in Shinya et al. (1999, Biomed & Pharmacother, 53, 471) where the fusion proteins were expressed in 293 cells.

Shinya et al. further suggest that the pharmacokinetic properties of fusion proteins containing the C4bp core domain are modified due to the increase in the *in vivo* plasma half-life of these recombinant fusion proteins in mice. As the core domain used is of human origin, adverse immunological consequences from its administration to humans would be minimised.

To date, fusion proteins based on C4bp core protein have been expressed in eukaryotic cells. The yields of fusion protein from eukaryotic cells has rarely reached 2 micrograms per millilitre of culture supernatant (Oudin et al. *ibid*) and this could be achieved only after rounds of gene amplification. This level is too low for the economic production of large quantities of many fusion protein for therapeutic use.

One possible way of achieving higher yields would be to use a prokaryotic expression system. WO91/00567 suggests that prokaryotic host cells may be used in the production of C4bp-based proteins, though there is no experimental demonstration of any such production. A number of considerations however, would suggest that the use of prokaryotic systems would be disadvantageous. In particular, many eukaryotic proteins lose some or all of their active folded structure when expressed in cells such as *Escherichia coli*. Other eukaryotic proteins denature or are completely inactive when expressed in prokaryotic cells.

C4bp is a secreted protein in mammals, and these are known in the art to be particularly difficult to produce in a correctly folded form in prokaryotes. Proteins with disulphide bridges are particularly problematic, as are those that require oligomerisation. Disulphide bonds are not normally produced in the reducing environment of the bacterial cytoplasm, and when they can form, they can stabilise misfolded or aggregated forms of the protein.

Usually, recombinant proteins expressed in prokaryotes are aggregated inside inclusion bodies within the host prokaryotic cell. These are discrete particles or globules separate from the rest of the cell which contain the expressed proteins usually in an agglomerated or inactive form. The presence of the expressed protein in the inclusion bodies makes it difficult to recover the protein in active soluble form as the necessary refolding techniques are techniques are inefficient and costly. Proteins purified from inclusion bodies have to be laboriously manipulated, denatured and refolded to obtain active functional proteins at relatively poor yields.

With regard to expressing C4bp core fusion proteins in prokaryotic cells, other considerations have also to be taken into account. Firstly, each core monomer retains two cysteine residues, and according to the model of C4bp multimers accepted in the art, these cysteines are required to form inter-molecular disulphide bonds during the assembly of multimers. The reducing environment of the prokaryotic cytosol such as the bacterial cytosol would be expected to prevent the formation of C4bp core multimers by reducing these disulphide bonds.

Secondly, multimers are assembled during passage through the eukaryotic secretion apparatus, which is known to assist protein folding in ways not available in prokaryotes (e.g. the presence of protein disulphide isomerase and unique chaperones). Thirdly, even under conditions where relatively small yields were obtained in eukaryotic cells (micrograms per millilitre), this secretory pathway is unable to produce homogenous protein.

Summary of the Invention.

The inventors have surprisingly found that fusion proteins of C4bp core are not only efficiently synthesized in prokaryotic cells but that the C4bp core itself is capable of folding correctly, and assembling into homogeneous multimers in the reducing environment of the prokaryotic cytosol. The multimers of C4bp core which are produced in prokaryotic cells surprisingly have been found to contain disulphide bonds.

Further, the inventors have also found that proteins fused to the C4bp core produced in the prokaryotic expression systems retain their functional activity. The present invention

therefore provides a method for obtaining a recombinant fusion protein comprising a scaffold of a C-terminal core protein of C4bp alpha chain, said recombinant fusion protein being capable of forming multimers in soluble form in the cytosol of a prokaryotic host cell, the method including the steps of

- (i) providing a prokaryotic host cell carrying a nucleic acid encoding said recombinant protein operably linked to a promoter functional in said prokaryotic cell;
- (ii) culturing the host cell under conditions wherein said recombinant protein is expressed; and
- (iii) recovering the recombinant protein wherein said protein is recovered in multimeric form without performing a scaffold refolding step.

We have found that the yield of protein in cell cultures of the invention can be relatively high, for example greater than 2 mg/l of culture, such as greater than 5 mg/l of culture, preferably greater than 10 mg/l of culture, such as greater than 20 mg/l culture, and even more preferably greater than 100 mg/l culture.

C4bp core fusion proteins of the invention comprise a C4bp core protein sequence fused, at the N- or C-terminus, to a biologically active sequence of interest.

Description of the Drawings.

Figure 1 shows an alignment of C4bp sequences from different species.

Figure 2 shows purification of the fusion protein db-C4bp (where db is a peptide described in Example 1) from an ion-exchange column.

Figure 3 shows further purification of db-C4bp on a second ion-exchange column.

5 Figure 4 shows purification of db-C4bp on a gel chromatography column.

Figure 5 shows purification of db-C4bp on an ion-exchange column following a heating step.

10 Figure 6 shows further purification of db-C4bp on a gel chromatography column.

Figure 7 shows the activity of DsbA-C4bp in an insulin assay.

15 Figure 8 shows the sequence of the promoter and C4bp coding region in pAVD77.

20 Figure 9 shows analysis of C4bp fusion proteins under non-reducing conditions.

Detailed Description of the Invention.

Core protein of C4bp alpha chain.

25 This is referred to herein as the "C4bp core protein" or "core protein", or "C4bp scaffold". The terms are used interchangeably. This protein may be a mammalian C4bp core protein or a fragment thereof capable of forming multimers, or a synthetic variant thereof capable of forming multimers.

30 The sequences of a number of mammalian C4bp proteins are available in the art. These include human C4bp core protein (SEQ ID NO:1). There are a number of homologues of human C4bp

core protein available in the art. There are two types of
homologue: orthologues and paralogues. Orthologues are defined
as homologous genes in different organisms, i.e. the genes
share a common ancestor coincident with the speciation event
5 that generated them. Paralogues are defined as homologous
genes in the same organism derived from a gene, chromosome or
genome duplication, i.e. the common ancestor of the genes
occurred since the last speciation event.

10 For example, a search of GenBank indicates mammalian C4bp core
homologue proteins in species including rabbit, rat, mouse and
bovine origin (SEQ ID NO:2-5 respectively). Paralogues have
been identified in pig (ApoR), guinea pig (AM67) and mouse
(ZP3); shown as SEQ ID NO:6-8 respectively.

15 An alignment of SEQ ID NOS:1-8 is shown as Figure 1. It can
be seen that all eight sequences have a high degree of
similarity, though with a greater degree of variation at the
C-terminal end. Further C4bp core proteins may be identified
20 by searching databases of DNA or protein sequences, using
commonly available search programs such as BLAST.

Where a C4bp protein from a desired mammalian source is not
available in a database, it may be obtained using routine
25 cloning methodology well established in the art. In essence,
such techniques comprise using nucleic acid encoding one of
the available C4bp core proteins as a probe to recover and to
determine the sequence of the C4bp core proteins from other
species of interest. A wide variety of techniques are
30 available for this, for example PCR amplification and cloning
of the gene using a suitable source of mRNA (e.g. from an
embryo or an actively dividing differentiated or tumour cell),
or by methods comprising obtaining a cDNA library from the

mammal, e.g. a cDNA library from one of the above-mentioned sources, probing said library with a known C4bp nucleic acid under conditions of medium to high stringency (for example 0.03M sodium chloride and 0.03M sodium citrate at from about 50°C to about 60°C), and recovering a cDNA encoding all or part of the C4bp protein of that mammal. Where a partial cDNA is obtained, the full length coding sequence may be determined by primer extension techniques.

A fragment of a C4bp core protein capable of forming multimers may comprise at least 47 amino acids, preferably at least 50 amino acids. The ability of the fragment to form multimers may be tested by expressing the fragment in a prokaryotic host cell according to the invention, and recovering the C4bp fragment under conditions which result in multimerisation of the full 57 amino acid C4bp core, and determining whether the fragment also forms multimers. Desirably a fragment of C4bp core comprises at least residues 6-52 of SEQ ID NO:1 or the corresponding residues of its homologues.

The human C4bp core protein of SEQ ID NO:1 corresponds to amino acids +493 to +549 of full length C4bp protein sequence. A fragment of this known in the art to form multimers corresponds to amino acids +498 to +549 of C4bp core protein.

Variants of C4bp core and fragments capable of forming multimers, which variants likewise retain the ability to form multimers (which may be determined as described above for fragments) may also be used. The variant will preferably have at least 70%, more preferably at least 80%, even more preferably at least 90%, for example at least 95% or most preferably at least 98% sequence identity to a wild type mammalian C4bp core or a multimer-forming fragment thereof.

In one aspect, the C4bp core will be a core which includes the two cysteine residues which appear at positions 6 and 18 of SEQ ID Nos:1-3 and 5-8. Desirably, the variant will retain the relative spacing between these two residues.

5

The above-specified degree of identity will be to any one of SEQ ID NOS:1-8 or a multimer-forming fragment thereof.

10

Most preferably the specified degree of identity will be to SEQ ID NO:1 or a multimer-forming fragment thereof.

15

The degree of sequence identity may be determined by the algorithm GAP, part of the "Wisconsin package" of algorithms widely used in the art and available from Accelrys (formerly Genetics Computer Group, Madison, WI). GAP uses the Needleman and Wunsch algorithm to align two complete sequences in a way that maximises the number of matches and minimises the number of gaps. GAP is useful for alignment of short closely related sequences of similar length, and thus is suitable for determining if a sequence meets the identity levels mentioned above. GAP may be used with default parameters.

20

25

Synthetic variants of a mammalian C4bp core protein include those with one or more amino acid substitutions, deletions or insertions or additions to the C- or N-termini. Substitutions are particularly envisaged. Substitutions include conservative substitutions. Examples of conservative substitutions include those set out in the following table, where amino acids on the same block in the second column and preferably in the same line in the third column may be substituted for each other:

30

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y
OTHER		N Q D E

Examples of fragments and variants of the C4bp core protein which may be made and tested for their ability to form multimers thus include SEQ ID NOs: 9 to 16, shown in Table 1 below:

A	B	C
9	-----CEQVLTGKRLMQCLPNPEDVKMALEVYKLSLEIEQLELQDSARQSTLDKEL	100
10	ETPEGCEQVLTGKRLMQCLPNPEDVKMALEVYKLSLEIKQLELQDSARQSTLDKEL	98
11	-----CEQVLTGKRLMQCLPNPEDVKMALEVYKLSLEIKQLELQDSARQSTLDKEL	98
12	ETPEGCEQVLTGKRLMQCLPNPEDVKMALEIYKLSLEIEQLELQDSARQSTLDKEL	98
13	ETPEGCEQVLTGKRLMQCLPNPEDVKMALEIYKLSLEIKQLELQDSARQSTLDKEL	96.5
14	---EGCEQALTGKRLMQCLPNPEDVKMALEIYKLSLEIKQLELQDSARQSTL----	94
15	ETPEGSEQVLTGKRLMQSLPNPEDVKMALEVYKLSLEIKQLELQDSARQSTLDKEL	94
16	---EGSEQALTGKRLMQSLPNPEDVKMALEIYKLSLEIEQLELQDSARQSTLDK--	92.3

A=SEQ ID NO;; B= sequence, C= % identity, calculated by reference to a fragment of SEQ ID NO:1 of the same length.

Where deletions of the sequence are made, apart from N- or C-terminal truncations, these will preferably be limited to no more than one, two or three deletions which may be contiguous or non-contiguous.

Where insertions are made, or N- or C-terminal extensions to the core protein sequence, these will also be desirably limited in number so that the size of the core protein does not exceed the length of the wild type sequence by more than 20, preferably by more than 15, more preferably no more than

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10, amino acids. Thus in the case of SEQ ID NO:1, the core protein, when modified by insertion or elongation, will desirably be no more than 77 amino acids in length.

5 N- or C-terminal extensions may include flexible linkers such as (Gly-Gly-Gly-Gly-Ser)_n (where n is from 1 to 4) used in the art to attach protein domains (particularly antibody V domains) to each other.

10 When the fusion proteins of the invention are made by chemical synthesis, N- or C-terminal extensions may include analogues of amino acids not naturally present in proteins which can be used in the art of peptide and polypeptide synthesis.

15 **Recombinant protein.**

The recombinant protein of the invention will comprise a C4bp core (or "scaffold") as described above either alone or linked in-frame to at least one sequence of biological interest. Such a sequence may comprise a tag useful for identification or purification of the protein, and/or a protein useful in therapy, particularly human therapy.

25 The recombinant protein can be described as having a general structure of the formula: Bi_N-Co-Bi_C in which Co is the core protein as described above, and Bi_N is either the amino terminus of the core protein or at least one sequence (for example one or two) of biological interest, and Bi_C is either the C-terminus of the core protein or at least one sequence (for example one or two) of biological interest.

30 Preferably, one of Bi_N and Bi_C is not a sequence of biological interest (i.e. one or other is a terminal of the fusion or optionally a tag, such as a polyhistidine tag, to aid recovery

of the protein). More preferably, the biological sequence of interest is represented by B_i .

Alternatively, a protein or non-protein product of interest
5 may be coupled by synthetic means to a side-chain of the core, e.g. through the amino group of the side-chain of a lysine residue or through cysteine residues added within, or at the end of, the core sequence; or to the existing cysteine residues.

10 It is preferred that the biological sequence of interest is not all or part of a C4 binding protein normally linked to the C4bp core protein, i.e., the biological sequence of interest is a heterologous sequence.

15 We have found that proteins falling within the above definition can be expressed in and recovered from bacterial expression systems in multimeric form without the need for scaffold refolding. We have expressed proteins which have a
20 monomer weight up to about 30 kDa. The invention may thus be used to express proteins in this size range, and more generally for proteins up to about 100 kDa, more preferably about 50 kDa.

25 A particular class of fusion proteins will be those in which the C4bp core is fused to a peptide of from 2 to 25 amino acid residues. Many biologically active peptides are known or can be selected through phage display. However, they are often unstable in vivo, not least because they can be filtered
30 through the renal glomerulus. Fusing them to the core scaffold makes filtration impossible. In addition, it confers avidity on the oligomerised peptides (such that they bind their targets more tightly and are effective at lower doses, and can

cross-link receptors). Particular biologically active peptides of interest include naturally occurring peptide or polypeptide hormones, such as somatostatin, calcitonin and alpha-MSH (melanocyte stimulating hormone) and variants thereof as well as other mentioned elsewhere herein.

Thus a range of fusion proteins of C4bp core protein may synthesized using the method of the present invention. The multimeric fusion proteins produced will be expected to exhibit increased bioactivity because multimers will have a higher density of the moiety attached to the C4bp core protein and would thus be expected to have a longer half life and an decreased turnover rate.

The sequence(s) of biological interest may be a polypeptide or a chemical compound (e.g. a drug or pro-drug) or a carbohydrate which is heterologous to the C4bp core protein used in the invention. In other words, it is not part of the same molecule in nature. It may be derived from the same organism. When the attached moiety is a chemical compound, the attachment may serve to protect the compound from metabolism and excretion, for example by hepatic cytochromes, as well as serving to deliver it to tissues. Examples of polypeptides include those used for medical or biotechnological use, such as insulin, cytokines including interleukins and interferons, antibodies and their fragments, growth factors, receptors, receptor ligands, agonists or antagonists, enzymes, enzyme antagonists, antigens, toxins and proteases.

Fusion proteins prepared according to the invention, and the novel fusion proteins of the invention described herein, may be prepared in the form of a pharmaceutical composition which

comprises the protein together with one or more pharmaceutically acceptable carriers or diluents. The composition will be prepared according to the intended use and route of administration of the fusion protein.

5

Pharmaceutically acceptable carriers or diluents include those used in formulations suitable for oral, rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous, intradermal, intrathecal and epidural) administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy.

For solid compositions, conventional non-toxic solid carriers include, for example, pharmaceutical grades of mannitol, lactose, cellulose, cellulose derivatives, starch, magnesium stearate, sodium saccharin, talcum, glucose, sucrose, magnesium carbonate, and the like may be used. The active compound as defined above may be formulated as suppositories using, for example, polyalkylene glycols, acetylated triglycerides and the like, as the carrier. Liquid pharmaceutically administrable compositions can, for example, be prepared by dissolving, dispersing, etc, a fusion protein of the invention optional pharmaceutical adjuvants in a carrier, such as, for example, water, saline aqueous dextrose, glycerol, ethanol, and the like, to thereby form a solution or suspension. If desired, the composition to be administered may also auxiliary substances such as pH buffering agents and the like. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in this art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 19th Edition, 1995.

The composition or formulation to be administered will, in any event, contain a quantity of the active compound(s) in an amount effective to alleviate the symptoms of the subject being treated. Dosage forms or compositions containing active ingredient in the range of 0.25 to 95% with the balance made up from non-toxic carrier may be prepared.

Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. A more recently devised approach for parenteral administration employs the implantation of a slow-release or sustained-release system, such that a constant level of dosage is maintained. See, e.g., US Patent No. 3,710,795.

The following classes of polypeptides are preferred, but the invention is not limited thereto:

Cytokines

Interleukins include any known interleukin including IL-1, IL-2, IL-3, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 and IL-12. Interleukins are modulators of the immune system. Some interleukins are involved in the inflammatory response or in the immune response to disease.

Interferons include any form of IFN-alpha, as well as IFN-beta and IFN-gamma. These also have use in modulation of the immune response.

A further class of cytokines are the tumour necrosis factors TNF-alpha and TNF-beta.

Other cytokines include members of the MIP family including MIP-1 α , MIP-1 β and RANTES. RANTES binds the CCR5 HIV co-receptor and therapy with RANTES may be effective in alleviating the progression of HIV infection.

Antibodies

The affinity of antibodies or antibody fragments for antigens may be increased by oligomerisation when the antibodies are produced as C4bp core fusion proteins according to the method of the present invention. Antibody fragments may be fragments such as Fv, Fab and F(ab')₂ fragments or any derivatives thereof, such as a single chain Fv fragments. The antibodies or antibody fragments may be non-recombinant, recombinant or humanised. The antibody may be of any immunoglobulin isotype, e.g., IgG, IgM, and so forth.

In another aspect, the antibody fragments may be camelised V_H domains. It is known that the main intermolecular interactions between antibodies and their cognate antigens are mediated through V_H CDR3. However, V_H-only antibodies, such as those derived from camel or llama (naturally V_H-only single chain antibodies), have only low affinity for cognate antigen.

The method of the present invention makes it possible to obtain improved yields of oligomers of C4bp core proteins with V_H domains, or V_H CDR3 domains which are high-affinity antibodies. Two or more domains may be included to the C4bp core oligomer made according to the method of the present invention; up to 8 domains may be included, forming an octameric antibody molecule.

Antibody targets may include tumour-associated antigens, including CEA and erbB, which are found in many colon and breast tumours respectively.

5

In one embodiment, the biological protein of interest may comprise the antibody fused to an enzyme capable of converting a prodrug into a drug toxic to the tumour cell. This can be used in a method of antibody-directed enzyme-prodrug therapy (ADEPT). Alternatively, monomers of carrying a tumour directed antibody and monomers carrying such an enzyme (e.g. a carboxypeptidase, a nitroreductase or the like) may be co-expressed in a cell or expressed in separate cells and mixed together to form heteromultimers directed to a tumour cell.

15

Antibodies may also be targeted to antigens of pathogenic organisms, including those mentioned below in the context of antigens for use as immunogens.

20

Growth factors

Growth factors include hormones such as growth hormone (in particular human growth hormone, hGH, as well as monocyte colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), erythropoietin and platelet derived growth factor (PDGF). Active fragments of such growth factors may also be used. Mammalian, particularly human, growth factors are particularly preferred.

25

Receptors

Receptors may be useful therapeutically in binding to proteins in the human body which are expressed at aberrant or unwanted levels.

For example, over-expression of TNF-alpha is associated with rheumatoid arthritis, and anti-TNF therapy has been successful in treatment of this condition. The biological protein of interest may thus be a TNF-alpha receptor.

A receptor of interest is also another member of the TNF receptor family, known as the BAFF receptor (Thompson et al. Science, 2001, 293, 2108). The human BAFF receptor (Genbank Accession no. AF373846) is a 184 amino acid protein which binds the TNF-related ligand BAFF. Over-expression of this ligand in mice can cause a systemic lupus erythematosus (SLE)-like symptom, and thus the BAFF receptor is of interest as a possible therapeutic of this disease.

In one aspect, the invention provides a fusion protein of the C4bp core and a BAFF receptor, including fragment of the extracellular domain thereof capable of binding a BAFF ligand. Such a fragment may correspond to amino acids 2-51 of BAFF.

Cell surface receptors are also of interest. For example, CD4 receptor is a target for the HIV surface protein gp120/160, and it has been widely proposed in the art to use CD4, or a soluble fragment thereof, as a therapeutic for HIV infection such that the CD4 blocks the ability of circulating HIV to enter CD4+ T-cells.

Other cell surface receptors are also associated with viral infection, for example CD46 with measles virus (Christiansen et al, *ibid*), and such cell surface receptor proteins may also be used in the present invention.

Receptor ligands, agonists or antagonists

Many cell surface receptors are activated by dimerisation. Well known examples are those for insulin and erythropoietin. The function of the ligand is to bind simultaneously to two
5 receptors, thus dimerising and activating them. In the examples cited, receptor autophosphorylation occurs. This activates the receptor, which has a tyrosine kinase domain in its intracellular portion. The kinase is inactive when the receptor is monomeric, but is activated on dimerisation. This
10 triggers a cascade of intracellular events, collectively referred to as signal transduction.

Whilst some ligands, such as substance P, are short polypeptides, others (including insulin (51 amino acids) as
15 well as kinase and phosphatase substrates) are complex molecules which possess binding loops projecting from the surface thereof. Smaller molecules which can mimic the natural ligands for receptors are useful for research purposes (for example to understand the specificity of ligand receptor
20 binding).

Short peptides or loops may be incorporated into fusion proteins according to the present invention to form a polyvalent receptor ligand or kinase / phosphatase substrate,
25 useful for activating or inhibiting receptors and/or kinases at very low concentrations.

Variation may be introduced into the heterologous polypeptides inserted onto the scaffold in order to map the specificity of
30 receptors or kinases/phosphatases for their ligands or substrates. Variants may be produced of the same loop, or a set of standard different loops may be devised, in order to assess rapidly the specificity of a novel kinase/phosphatase.

Variants may be produced by randomisation of sequences according to known techniques, such as PCR. They may be subjected to selection by a screening protocol, such as phage display, before incorporation into protein scaffolds in accordance with the invention.

Agonists include peptides, including peptide mimetics, which bind to a receptor so as to trigger the action of the receptor in even in the absence of the natural ligand for that receptor. An example of an agonist is the thrombopoietin agonist peptide. This linear 14-mer peptide is found to be 4,000-fold more active when dimeric than when monomeric (Dower W.J. et al. Stem Cells (1998) 16, Suppl 2, 21 Peptide agonists of the thrombopoietin receptor). Fusion of this sequence, IEGPTLRQWLAARA, to the core domain of C4bp, as described below for other peptides should create a very potent thrombopoietin agonist, useful for promoting platelet production and/or maturation.

In a further aspect, the invention provides a recombinant protein comprising a C4bp core protein and a thrombopoietin agonist peptide, and the use of such a protein in a method of therapy for promoting platelet production and/or maturation in a human subject. The method comprises administering to a subject in need of treatment an effective amount of the protein.

A further example of an agonist is the somatostatin peptide. This cyclic peptide is known to bind to a number of G-protein coupled receptors, and to inhibit the release of somatotropin. An analogue is marketed as Sandostatin (Novartis) for a number of medical indications, including the treatment of side

effects associated with malignant carcinoid tumours and the treatment of diarrhea caused by gastrointestinal infections.

5 Fusion of the somatostatin sequence to filamentous bacteriophage, as described in the British Journal of Pharmacology (1998) "Somatostatin displayed on filamentous phage as a receptor-specific agonist". Volume 125, pages 5-16, produces a hybrid phage capable of binding to and activating somatostatin receptors. Fusion of somatostatin to the C4bp scaffold (with the scaffold replacing the phage) similarly produces an avid agonist for somatostatin receptors, which has more desirable properties as a medicament than hybrid phage. Similarly, the oligomeric agonist so produced is capable of oligomerising the somatostatin receptors, which may enhance signalling, as described by Patel et al. in *Proc.Natl.Acad. Sci. USA* (2002) Volume 99, pages 3294-3299.

Thus the invention provides a means to prepare a recombinant fusion protein as set out above wherein said fusion protein comprises somatostatin. The invention further provides a fusion protein of a C-terminal core protein of C4bp alpha chain linked to somatostatin. The invention further provides the use of this protein or nucleic acid vectors (as further defined and described herein) encoding this protein in a method of treatment, including the treatment of side effects associated with malignant carcinoid tumours and the treatment of diarrhea caused by gastrointestinal infections.

Antagonists include peptides which bind to receptors and block the natural ligand from binding.

Enzymes

Numerous biological reactions involve the sequential, and/or synergistic, action of a plurality of protein activities. Such protein activities may be incorporated into a single molecule in accordance with the present invention.

Preferably, therefore, the monomers which are used to compose the oligomer according to the invention incorporate amino acid sequences which encode distinct biological activities. The activities are advantageously complementary, such that they are required sequentially in a biological reaction, or act synergistically. The invention therefore provides plurifunctional macromolecular structures comprising one or more enzymes.

Examples of enzymes include bacterial enzymes such as DsbA of *E. coli*.

Antigens

A particular use for multimers of produced in accordance with the invention is in the production of immunogens (this term is used interchangeably herein with "antigens"). A major application of this C4bp core fusion protein scaffold technology produced following the method of the present invention is the use of the assembled or multimerised peptides or polypeptides as antigens. The oligomerisation improves both detection of antibodies against, and the induction of antibodies to, such antigens. Some of these antigens may be of prophylactic value; they might be useful for vaccination. The method allows rapid progress from nucleotide sequences to the production of recombinant antigens in a polyvalent form. Predicted open reading frames (ORFs) can be used to design oligonucleotide sequences encoding the predicted protein sequence. Cloning of these oligonucleotides into the vectors

encoding the C4bp core protein allows a very rapid production of antigens, without, for example the need for isolating cDNAs and expressing them in heterologous systems such as *E. coli*.

5 Bacterial immunogens, parasitic immunogens and viral immunogens are useful as polypeptide moieties to create multimeric or hetero-multimeric C4bp fusion proteins useful as vaccines.

10 Bacterial sources of these immunogens include those responsible for bacterial pneumonia, pneumocystis pneumonia, meningitis, cholera, tetanus, tuberculosis and leprosy.

Parasitic sources include malarial parasites, such as
15 Plasmodium.

Viral sources include poxviruses, e.g., cowpox virus and orf virus; herpes viruses, e.g., herpes simplex virus type 1 and 2, B-virus, varicellazoster virus, cytomegalovirus, and
20 Epstein-Barr virus; adenoviruses, e.g., mastadenovirus; papovaviruses, e.g., papillomaviruses such as HPV16, and polyomaviruses such as BK and JC virus; parvoviruses, e.g., adeno-associated virus; reoviruses, e.g., reoviruses 1, 2 and 3; orbiviruses, e.g., Colorado tick fever; rotaviruses, e.g.,
25 human rotaviruses; alphaviruses, e.g., Eastern encephalitis virus and Venezuelan encephalitis virus; rubiviruses, e.g., rubella; flaviviruses, e.g., yellow fever virus, Dengue fever viruses, Japanese encephalitis virus, Tick-borne encephalitis virus and hepatitis C virus; coronaviruses, e.g., human
30 coronaviruses; paramyxoviruses, e.g., parainfluenza 1, 2, 3 and 4 and mumps; morbilliviruses, e.g., measles virus; pneumovirus, e.g., respiratory syncytial virus; vesiculoviruses, e.g., vesicular stomatitis virus;

lyssaviruses, e.g., rabies virus; orthomyxoviruses, e.g., influenza A and B; bunyaviruses e.g., LaCrosse virus; phleboviruses, e.g., Rift Valley fever virus; nairoviruses, e.g., Congo hemorrhagic fever virus; hepadnaviridae, e.g., hepatitis B; arenaviruses, e.g., lcm virus, Lasso virus and Junin virus; retroviruses, e.g., HTLV I, HTLV II, HIV-1 and HIV-2; enteroviruses, e.g., polio virus 1,- 2 and 3, coxsackie viruses, echoviruses, human enteroviruses, hepatitis A virus, hepatitis E virus, and Norwalk- virus; rhinoviruses e.g., human rhinovirus-; and filoviridae, e.g., Marburg (disease) virus and Ebola virus.

Antigens from these bacterial, viral and parasitic sources may be used in the production of multimeric proteins useful as vaccines. The multimers may comprise a mixture of monomers carrying different antigens.

Immunogens to human proteins for research or therapeutic purposes may be made. Immunogenic peptides, capable of raising an immune response when exposed to the immune system of an organism, are preferred polypeptides for making C4bp core protein fusion proteins following the method of the invention. The improved yield of oligomerised C4bp core fusion proteins from the present invention has many applications not only in vaccination but also in research. For example, the generation of human gene sequence data by the human genome project has made the generation of antisera reactive to new polypeptides a pressing requirement. The same requirement applies to prokaryotic, such as bacterial, and other eukaryotic, including fungal, gene products. Immunogens of interest fused to C4bp core multimers are thought to have increased efficiency due to their increased avidity for immunoglobulin molecules.

The present invention has many advantages in the generation of an immune response. For example, the use of oligomers can permit the presentation of a number of antigens, simultaneously, to the immune system. This allows the preparation of polyvalent vaccines, capable of raising an immune response to more than one epitope, which may be present on a single organism or a number of different organisms. Thus, vaccines formed according to the invention may be used for simultaneous vaccination against more than one disease, or to target simultaneously a plurality of epitopes on a given pathogen. The epitopes may be present in a single monomer units or on different monomer units which are combined to provide a heteromultimer.

Moreover, the invention may be exploited by incorporating an adjuvant on the C4bp core oligomer, together with the immunogen. Suitable adjuvants are, for example, bacterial toxins and cytokines, such as interleukins. The potency of the immunogen is thereby increased, allowing more efficient raising of antisera and more efficient immunisation. A highly preferred adjuvant is the C3d component of complement.

Having C4bp core fusion proteins is useful in the context of immunisations, because the core protein is not only present normally in the serum or plasma of the recipient of the immunisation, but also because it does not itself evoke an immune response. C4bp proteins are known in a number of mammalian species, and the appropriate homologues for mammalian species may be found by those skilled in the art using standard gene cloning techniques.

The fact that this system allows production of soluble protein in *E. coli* enables using it to produce, as folded soluble proteins, domains or fragments of proteins that would not fold when expressed on their own due to a lack of constraint on their C-terminal and /or N-terminal ends. Engineering a specific cleavage site enables production of the free domain of interest. Similarly constraining the N-terminal and/or C-terminal end of a peptide of interest could be beneficial during refolding processes. Furthermore, as the oligomerisation structure is very resistant to denaturation and to disassembly, it would be stable during denaturation of the inserted protein. Therefore, during refolding, for an equal amount of protein of interest, the actual concentration of free protein would be diminished by a factor equal to the oligomerisation number. Oligomerisation may also be beneficial for purification purposes as many methods in protein technology are not optimised to work with proteins and specifically peptides of very low molecular weight.

Assay methods

The C4bp core fusion proteins produced following the method of the invention may be applied to the detection or the neutralisation of antibodies *in vivo* or *in vitro*. For example, *in vitro* polyvalent or monovalent antigen-bearing C4bp core fusion proteins may be used to select antibody molecules derived from phage display experiments. Moreover, *in vivo*, antigen-bearing C4bp core fusion proteins produced according to the method of invention may be used to neutralise autoantibodies in autoimmune disease, or to detect antibodies which may be indicative of pathological conditions, such as in HIV testing or other diagnostic applications.

Phage Display

Phage display technology has proved to be enormously useful in biological research. It enables ligands to be selected from large libraries of molecules. The proteins of the present invention also harnesses the power of this technique, but with some powerful advantages over normal applications. C4bp molecules can be displayed as monomers on fd bacteriophages, just as single-chain Fv molecules are. Libraries of fusions are constructed by standard methods, and the resulting libraries screened for ligands of interest. It is important to note that this is an affinity based selection. After characterisation, the ligands selected for affinity, can be oligomerised, and thus take advantage of avidity. When the target for the ligand is oligomeric, very tight binding will result. Furthermore, ligands selected as monomers, will be able to cross-link or oligomerise their binding partners. An application of this effect is in triggering receptor activation.

Protein chips

Currently, DNA microarrays, whether of oligonucleotides, PCR products or cloned DNAs, are major tools enabling rapid development in the highly parallel analysis of gene expression. Clearly, in many situations, it would be far preferable to monitor gene expression directly, that is, by assaying protein expression levels rather than mRNA levels. The latter are but an indirect measure of gene activity which rely on the hybridisation of labelled cDNA and can be very misleading because there is often a poor correlation between the abundance of a particular mRNA and the frequency at which it is translated into proteins. In addition, mRNA analysis can not possibly determine whether the encoded protein, even

if translated, is active. This may depend on post-translational modification.

Thus protein arrays comprising fusion proteins of a core scaffold and a range of ligands for proteins of interest may be produced and used to determine levels of expression of those proteins in a sample.

For example, an array of bacterial cells expressing the scaffold-ligand fusions may be provided, such that the fusions are expressed and recovered *in situ*, followed by addition of the sample. Alternatively, the fusions may be produced separately and then arrayed on a suitable solid support to provide for detection of the proteins in the sample.

Detection may be by providing a predetermined amount of the proteins of interest labelled to compete against the proteins present in the sample, and measuring how much labelled protein binds to the ligand. Alternatively, the ligand may be labelled and the amount of labelled ligand bound to the protein of interest detected.

Nucleic Acids

Proteins comprising the C4bp core are produced by expression of the protein in a prokaryotic host cell, using a nucleic acid construct encoding the recombinant protein.

The construct will generally be in the form of a replicable vector, in which sequence encoding the protein is operably linked to a promoter suitable for expression of the protein in a desired host cell. The promoter may be an inducible promoter. Suitable promoters include the T7 promoter, the *tac* promoter, the *trp* promoter, the lambda promoters P_L or P_R and others well known to those skilled in the art.

The vectors may be provided with an origin of replication and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an antibiotic resistance gene such as an ampicillin, tetracycline or preferably kanamycin resistance gene. There are a wide variety of bacterial expression vectors known as such in the art, and the present invention may utilise any vector according to the individual preferences of those of skill in the art.

A wide variety of prokaryotic host cells can be used in the method of the present invention. These hosts may include strains of Escherichia, Pseudomonas, Bacillus, Lactobacillus, Thermophilus, Salmonella, Enterobacteriaceae or Streptomyces. For example, if *E. coli* from the genera Escherichia is used in the method of the invention, preferred strains of this bacterium to use would include BL21(DE3) and their derivatives including C41(DE3), C43(DE3) or CO214(DE3), or other strains resistant to the toxicity of recombinant protein expression as described and made available in WO98/02559.

Even more preferably, derivatives of these strains lacking the prophage DE3 may be used when the promoter is not the T7 promoter.

DNA vaccines and therapeutics

In another aspect, the invention provides a eukaryotic expression vector comprising a nucleic acid sequence encoding a recombinant fusion protein comprising a scaffold of a C-terminal core protein of C4bp alpha chain for the use in the treatment of the human or animal body.

Such treatment would achieve its therapeutic effect by introduction of a specific nucleic acid sequence into cells or tissues affected by a genetic or other disease, or by introduction of a nucleic acid sequence encoding an antigen
5 for the purposes of raising an immune response. It is also possible to introduce genetic sequences into a different cell or tissue than that affected by the disease, with the aim that the gene product will have direct or indirect impact on the diseases cells or tissues. Delivery of nucleic acids can be
10 achieved using a plasmid vector (in "naked" or formulated form) or a recombinant expression vector.

Various viral vectors which can be utilized for gene therapy include adenovirus, herpes virus, vaccinia or an RNA virus
15 such as a retrovirus. The retroviral vector may be a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukaemia virus (MoMuLV), Harvey murine sarcoma virus
20 (HaMuSV), murine mammary tumour virus (MuMTV), and Rous Sarcoma Virus (RSV). When the subject is a human, a vector such as the gibbon ape leukaemia virus (GaLV) can be utilized.

The vector will include a transcriptional regulatory sequence,
25 particularly a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer et al. 1982 J. Molec. Appl. Genet. 1, 273); the TK promoter of Herpes virus (McKnight, 1982 Cell 31,355); the
30 SV40 early promoter (Benoist et al.1981 Nature 290, 304); the Rous sarcoma virus promoter (Gorman et al. 1982 Proc. Natl Acad. Sci. USA 79, 6777); and the cytomegalovirus promoter (Foecking et al. 1980 Gene 45, 101).

Promoters specific for the cell type requiring the gene therapy are desirable in many instances. In a situation where a particular cell type is used as a platform to produce therapeutic proteins destined for another site (for either direct or indirect action), then the chosen promoter should work well in the "factory" site. Muscle is a good example for this, as it is post-mitotic, it could produce therapeutic proteins for years on end as long as there is no immune response against the protein-expressing muscle fibres.

Therefore, use of strong muscle promoters as are particularly applicable here. Except for treating a muscle disease per se, use of muscle is typically only suitable where there is a secreted protein so that it can circulate and function elsewhere (e.g., hormones, growth factors, clotting factors).

Administration of vectors of this aspect of the invention to a subject, either as a plasmid vector or as part of a viral vector can be affected by many different routes. Plasmid DNA can be "naked" or formulated with cationic and neutral lipids (liposomes) or microencapsulated for either direct or indirect delivery. The DNA sequences can also be contained within a viral (e.g., adenoviral, retroviral, herpesvirus, pox virus) vector, which can be used for either direct or indirect delivery. Delivery routes include but are not limited to intramuscular, intradermal (Sato, Y. et al. 1996 Science 273, 352), intravenous, intra-arterial, intrathecal, intrahepatic, inhalation, intravaginal instillation (Bagarazzi et al. 1997 J Med. Primatol. 26, 27), intrarectal, intratumour or intraperitoneal.

Thus the invention includes a vector as described herein as a pharmaceutical composition useful for allowing transfection of

some cells with the DNA vector such that a therapeutic polypeptide will be expressed and have a therapeutic effect (to ameliorate symptoms attributable to infection or disease). The pharmaceutical compositions according to the invention are prepared by bringing the construct according to the present invention into a form suitable for administration to a subject using solvents, carriers, delivery systems, excipients, and additives or auxiliaries. Frequently used solvents include sterile water and saline (buffered or not). One carrier includes gold particles, which are delivered biolistically (i.e., under gas pressure). Other frequently used carriers or delivery systems include cationic liposomes, cochleates and microcapsules, which may be given as a liquid solution, enclosed within a delivery capsule or incorporated into food.

An alternative formulation for the administration of gene therapy vectors involves liposomes. Liposome encapsulation provides an alternative formulation for the administration of polynucleotides and expression vectors. Liposomes are microscopic vesicles that consist of one or more lipid bilayers surrounding aqueous compartments. See, generally, Bakker-Woudenberg et al. 1993 Eur. J. Clin. Microbiol. Infect. Dis. 12, Suppl. 1, S61, and Kim, 1993 Drugs 46, 618. Liposomes are similar in composition to cellular membranes and as a result, liposomes can be administered safely and are biodegradable. Depending on the method of preparation, liposomes may be unilamellar or multilamellar, and liposomes can vary in size with diameters ranging from 0.02 μM to greater than 10 μM . See, for example, Machy et al. 1987 LIPOSOMES IN CELL BIOLOGY AND PHARMACOLOGY (John Libbey), and Ostro et al. 1989 American J. Hosp. Phann. 46, 1576.

Expression vectors can be encapsulated within liposomes using

standard techniques. A variety of different liposome compositions and methods for synthesis are known to those of skill in the art. See, for example, U.S. Pat. No. 4,844,904, U.S. Pat. No. 5,000,959, U.S. Pat. No. 4,863,740, U.S. Pat. No. 5,589,466, U.S. Pat. No. 5,580,859, and U.S. Pat. No. 4,975,282, all of which are hereby incorporated by reference.

In general, the dosage of administered liposome-encapsulated vectors will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Dose ranges for particular formulations can be determined by using a suitable animal model.

In one embodiment, the vector encodes a fusion protein comprising the core and, in addition, one or more antigens and optionally and preferably a protein with immunostimulatory properties. C3d is known to have strong immunostimulatory properties and may be used for this purpose, as may be an interleukin, particularly IL-2 or IL-12.

Cell culturing

Plasmids encoding fusion proteins in accordance with the invention may be introduced into the host cells using conventional transformation techniques, and the cells cultured under conditions to facilitate the production of the fusion protein. Where an inducible promoter is used, the cells may initially be cultured in the absence of the inducer, which may then be added once the cells are growing at a higher density in order to maximise recovery of protein.

Cell culture conditions are widely known in the art and may be used in accordance with procedures known as such.

Recovery of protein from culture

Once the cells have been grown to allow for production of the protein, the protein may be recovered from the cells. Because
5 we have found that surprisingly, the protein remains soluble, the cells will usually be spun down and lysed by sonication which keeps the protein fraction soluble and allows this fraction to remain in the supernatant following a further higher-speed (e.g. 15,000 rpm for 1 hour) centrifugation.

10 The fusion protein in the supernatant protein fraction may be purified further by any suitable combination of standard protein chromatography techniques. We have used ion-exchange chromatography followed by gel filtration chromatography.

15 Other chromatographic techniques, such as affinity chromatography, may also be used.

In one embodiment, we have found that heating the supernatant sample either after centrifugation of the lysate, or after any
20 of the other purification steps will assist recovery of the protein. The sample may be heated to about 70 - 80 °C for a period of about 10 to 30 minutes.

25 Depending on the intended uses of the protein, the protein may be subjected to further purification steps, for example dialysis, or to concentration steps, for example freeze drying.

The invention is illustrated by the following examples.

Example 1. Production of db-C4bp**Vector construct.**

An expression vector encoding the downstream box peptide sequence MASMNHKGS (Sprengert M.L., Fuchs E. and Porter A.G. 1996 "The downstream box: an efficient and independent translation initiation signal in *Escherichia coli*." EMBO J. Volume 15, 665-674) fused N-terminal to the 57 amino acid "core" domain of the human C4bp alpha chain was constructed.

Briefly, the C4bp core domain is encoded entirely within a single exon in the human genome, thus allowing it to be amplified directly from human genomic DNA. The oligonucleotide primers used were:

AVD102: 5'CCCGCGGATCCGAGACCCCGAAGGCTGTGA3'; and

AVD103: 5'CCCGGAATTCTTATTATAGTTCTTTATCCAAAGTGG3'.

These contained added restriction sites which were used for cloning the amplified DNA fragment. The 183 base-pair fragment obtained on digesting the PCR product with the enzymes BamHI and EcoRI was cloned downstream of the translational enhancer or "downstream box" and the T7 promoter in a plasmid vector.

The plasmid was derived from the plasmid pRsetA supplied by Invitrogen, but the fl origin of replication has been replaced by the par locus from the plasmid pSC101. It thus contains as functional elements: a selectable marker (ampicillin

resistance) an origin of replication (derived from the pUC family) and a T7 promoter and a T7 transcription terminator as well as the par locus. The resulting construct was designated plasmid pAVD 77. Figure 8 shows the sequence of the translational enhancer and T7 promoter fused to the coding sequence of C4bp (in small print).

The predicted size of the db-C4bp fusion protein is 7491.5 Da.

Transformation and expression.

The vector was transformed into the *E. coli* strain C41(DE3), a derivative (Bruno Miroux and John E. Walker 1996 "Over-
5 production of Proteins in Escherichia coli: Mutant Hosts that Allow Synthesis of some Membrane Proteins and Globular Proteins at High Levels." Journal of Molecular Biology Volume 260, 289-298) of BL21(DE3).

0 One litre of LB-Ampicillin medium was inoculated with the cells, which were incubated at 37°C with shaking for 3 hours (until OD600 nm reached 0.6) and then it was induced with IPTG (isopropylthiogalactoside) at a final concentration 0.7 mM for 3 hours. The cells were harvested by centrifugation at 4600
5 rpm for 30 min.

The pellet (P) was resuspended with 30 mls Tris 50 mM pH 7, and the cells were broken by sonication using an Emulsiflex apparatus twice (between each treatment, centrifugation at
10 15000 rpm for 1 hour, the supernatants from each spin (designated SN1 and SN2 respectively) were kept and the pellet P1 was resuspended with the same buffer).

Both supernatants were pooled (60 mls) and were split into two
15 solutions of 30 mls. Each of these 30 ml aliquots of the db-C4bp fusion protein was purified using one of two similar methods: these were identical except that a heating step in one method was replaced by a MonoQ ion-exchange step in the other.

10

Purification without a heating step

The native db-C4bp was purified from 500 mls of culture by ion-exchange chromatography (DEAE Fast Flow 70, using a column

of 13cm in height, and diameter of 2.6cm), using TrisHCl buffer (50mM pH7) and a salt gradient (0M - 1M NaCl). The fusion protein eluted between 300-400 mM NaCl. Fractions of 7.5 ml each were collected - see Figure 2.

5 Fractions B8 to B11 were pooled and dialyzed against TrisHCl 20 mM pH7. Then this solution was loaded on a ion-exchange column (MonoQ HR 16/10), using Tris buffer (50mM pH7) and a salt gradient (0M - 1M NaCl). Fractions of 2.5 ml were
10 collected. The fusion protein eluted between 500-550 mM NaCl (Figure 3).

Fractions A10 to B1 were pooled and the final solution was then concentrated to a volume of 10 mls before being
15 chromatographed on a gel filtration column (S75 26/60). Fractions of 5 ml were collected. The fusion protein was eluted from this column with a volume of 139 mls buffer (TrisHCl 100 mM pH7, 150 mM NaCl), see Figure 4. The
20 calibration of the column with molecular weight standards implies a molecular weight for this protein similar to albumin (67 kDa), which in Tris 50 mM + NaCl 150 mM also elutes with a volume of 139 mls, whereas the expected molecular weight of the monomer is 7.491 kDa. This indicates that the fusion
25 protein is oligomeric in structure when purified from the cytosol of *E. coli*, without any steps being taken to refold it.

Fractions A10 to B1 were pooled (312 µg/ml), and an aliquot was dialysed against sodium phosphate buffer, 100 mM, pH 7.4.

30 The protein yield per Litre of culture after purification was 12.4 milligrams.

The CD spectrum was examined and showed the presence of significant secondary structure, consistent with a properly folded protein complex.

5 Example 2. Purification of db-C4bp with a heating step

The solution containing the other 30 ml aliquot of db-C4bp was heated at 76°C for 15 minutes and then centrifuged at 20,500 rpm for 1 hour. The supernatant, containing db-C4bp, was purified by ion-exchange chromatography (DEAE Fast Flow 70 mls), using Tris buffer (50mM pH7) and a salt gradient (0M - 1M NaCl). Fractions of 7.5 ml were collected. The fusion protein eluted between 300-400 mM NaCl (Figure 5).

15 Fractions B8 to B11 were pooled and the final solution was then concentrated to a volume of 10 mls before being chromatographed on a gel filtration column (S-75 26/60). Fractions of 5 ml were collected. The fusion protein was eluted from this column with a volume of 140mls buffer (Figure 6). The calibration of the column with molecular weight standards implies a molecular weight identical to that of the protein purified without heating (see above), whereas the expected molecular weight of the monomer is 7.491 kDa. This fusion protein is therefore also oligomeric in structure when purified from the cytosol of *E. coli*, without any steps being taken to refold it. Furthermore, it remains oligomeric despite being heated to 76°C for 15 minutes in a buffer comprising 50 mM TrisHCl pH7 (i.e. no salt was present).

30 Fractions A11 to B1 were pooled (595.5 µg/ml) and an aliquot was dialysed against sodium phosphate (NaP) buffer 100 mM pH 7.4.

Analysis using circular dichroism showed that the spectrum obtained with the sample which had been subjected to heating was equivalent to that obtained using the unheated sample. This demonstrated that the secondary structure elements of the protein are retained despite heating.

The yield with the heating step was 3.5 milligrams per litre.

The addition of a heating step can significantly simplify the purification of proteins. In the example here, heating replaced one ion-exchange (MonoQ) step, and nevertheless resulted in a protein of at least equivalent purity.

Example 3: Treatment of protein with denaturant

To confirm further that the protein was indeed oligomeric, an attempt was made to denature purified protein in 6M guanadinium chloride and 20mM DTT (dithiothreitol) at room temperature before repeating gel filtration under denaturing conditions.

Briefly, a culture of 500mls of the cells of example 1 were grown and induced as described above. The fusion protein was purified by ion-exchange chromatography, using TrisHCl buffer (50mM pH 7.4) and a salt gradient (0M - 1M NaCl). The fusion protein eluted between 450-650 mM NaCl and was then concentrated to a volume of 10 mls. After this concentration step, the concentration of db-C4bp protein was 740 micrograms per ml.

The protein was then treated at a concentration of 740 micrograms per ml overnight at 4°C with 6M guanidinium chloride and 20 mM DTT before being chromatographed on a gel

filtration column (S-75). The fusion protein was eluted from this column with a volume of 11.4 mls buffer. Calibration of the column with molecular weight standards implies a molecular weight for this protein of approximately 60 kDa, whereas the expected molecular weight of the monomer is 7.5 kDa. This fusion protein is therefore oligomeric in structure when purified from the cytosol of *E. coli*, without any steps being taken to refold it and even when treated to denaturing conditions.

Repeating the denaturation step using 6M guanidine HCl for 2 or 16 hours and heating to 75°C-80°C did result in denatured protein, as evidenced by CD analysis.

Example 4: Cloning and recombinant expression in *E. coli* of the human C4bp core fused to a histidine tag sequence.

To demonstrate that the translational enhancer is not essential for high-level expression of the core domain in *Escherichia coli*, and to facilitate the purification of the protein, the DNA sequence encoding the downstream box was replaced by a sequence encoding a 6xHistidine tag by replacing an NdeI/BamHI restriction fragment in pAVD 77 with the following sequence:

CATATGCGGG GTTCTCATCA TCATCATCAT CATGGTCTGG TTCCGCGTGG ATCC

The resulting plasmid pAVD 93, overproduces a recombinant protein of 8.46 kDa with the following amino acid sequence:

MRGSHHHHHH GLVPRGSETP EGCEQVLTGK RIMQCLPNPE DVKMALEVYK
LSLEIEQLEL QRDSARQSTL DKEL

The plasmid pAVD 93 was transformed into the bacterial strain C41(DE3) and expression of the fusion protein was induced using IPTG as described in above. A protein of 8.5 kDa as shown by SDS-PAGE analysis was present in induced cultures 3 hours after induction but absent from uninduced cultures.

Example 5: Cloning and recombinant expression in *E. coli* of the human C4bp core fused to the DsbA protein

The fusion of the C4bp core domain to the short peptide sequences encoded by the downstream box enhancer or to the histidine tag does not necessarily imply that the fusion of the core domain to larger proteins is feasible. To determine this, the C4bp core was fused to the C-terminus of the DsbA protein, an enzyme normally found in the *E. coli* periplasmic space. DsbA comprises 177 amino acids, and as such, is substantially larger than the core domain itself (57 amino acids).

Construction of the plasmid pAVD 78, encoding the DsbA-C4bp fusion protein

The NdeI-BamHI DNA fragment in pAVD 77 encoding the downstream box enhancer was replaced by an NdeI-BamHI fragment encoding DsbA. The oligonucleotide primers used to obtain the fragment encoding DsbA were:

AVD52: 5'GGGGCCCCCATATGGCGCAGTATGAAGATGGTAAACAG3'; and
AVD115: 5'GGGGAATTCTTAGGATCCAGAACCTTTTTTCTCGGACAGATATTTTCAC3'.

These primers were used to amplify the DsbA coding sequence (lacking a stop codon) from the genomic DNA of *Escherichia coli*. The PCR product was digested with both NdeI and BamHI restriction enzymes, and cloned into pAVD 77 in to create pAVD 78.

The plasmid pAVD 77 was transformed into the bacterial strain C41(DE3) and expression of the fusion protein was induced using IPTG as described above. A protein of 28 kDa as shown by SDS-PAGE analysis was present in induced cultures 3 hours after induction, but absent from uninduced cultures. Surprisingly, this protein was present in the soluble fraction of the cell extract.

Purification of the DsbA-C4bp fusion protein

The fusion protein was purified by two ion-exchange chromatographic steps (first DEAE, secondly MonoQ), using Tris HCl buffer (50mM pH 7.4) and a salt gradient (0 M-1M NaCl) in each case. The fusion protein eluted after the first (DEAE) ion-exchange chromatography at approximately 100 mM NaCl and was then purified by a more resolute (MonoQ) ion-exchange chromatography. The fusion protein eluted at 350 mM NaCl from the MonoQ and was concentrated before being chromatographed on a S200 gel filtration column (10/30). The fusion protein was eluted from this column in a volume of 12.54 mls of buffer.

Calibration of the column with molecular weight standards implies a molecular weight for this protein of approximately 200 kDa. The expected molecular weight of the monomer is 28.08 kDa. This fusion protein is therefore also oligomeric in structure when purified from the cytosol of *E. coli*, without any steps being taken to refold it.

To verify that the fusion protein was indeed oligomeric, rather than that its behaviour on gel filtration was aberrant, the purified protein was denatured in 6M guanidinium chloride and 20 mM DTT (for 2 hours 30 minutes at room temperature) and the gel filtration repeated under denaturing conditions, (that is in the presence of 6M guanidinium chloride and 20 mM DTT).

Under these circumstance, the protein eluted in a volume of 12.5 mls, consistent with a molecular weight of approximately 220 kDa. The fusion protein is thus not denatured under these conditions: the protein is still oligomeric.

Complete denaturation of this protein was obtained after treatment with guanidine HCl for 16 hours at 4°C, in contrast to the Example 3 above, where heating to 75-80°C was required to obtain complete denaturation.

Activity of the DsbA-C4bp fusion protein

To test the activity, of DsbA-C4bp, an insulin assay was conducted. In the presence of DTT, active DsbA catalyses the reduction of insulin's disulphide bonds which enables the separation of the two chains, and thus provokes the precipitation of the free insulin B chain. A turbidimetric assay is thus used to detect the reduction of the disulphide bonds of insulin. (Holmgren A (1979) Thioredoxin catalyses the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J. Biol. Chem.* 254, 9627).

The final reaction mixture contains 0.14 mM freshly prepared insulin, 0.1 M potassium phosphate pH 7.0, 2 mM EDTA, and 0.67 mM DTT, and 100 µg of DsbA or 100 µg of DsbA-C4bp fusion protein in a final volume of 1.2 ml.

The reaction was initiated by addition of 8 µl of 0.1 M DTT and monitored by measuring the increase of turbidity at 650 nm every 5 minutes up to 60 minutes. Each sample was gently mixed 3-4 times prior to measuring the absorbance at 650 nm. The instrument blank of the reaction contained 0.1 M phosphate buffer pH 7.0, and 2 mM EDTA.

The results are shown in Figure 7, and demonstrate that the DsbA present in the DsbA-C4bp fusion protein is still active, and the activity is directly comparable to the activity of soluble DsbA.

Example 6: Analysis of C4bp fusion proteins under non-reducing conditions

The analysis of the db-C4bp fusion protein by polyacrylamide gel electrophoresis under denaturing but non-reducing conditions was conducted to determine the presence or absence of disulphide bonds between the monomers of the oligomer.

Aliquots (12 μ l) of db-C4bp (312 μ g/ml) were mixed with Laemmli buffer (Tris HCl 1.5 M pH 6.8, SDS 2%, glycerol 15%, 0.02% Bromophenol blue) with or without β -mercaptoethanol. These samples were boiled at 90°C for 5 min and analysed by electrophoresis through a 18% sodium dodecyl sulfate polyacrylamide gel, also lacking β -mercaptoethanol.

The result was that, in the absence of β -mercaptoethanol, the db-C4bp fusion protein migrated as an oligomer (in the top of the gel, Figure 9 right side) showing that disulphide bonds exist between the monomers. In contrast, the addition of β -mercaptoethanol resulted in the migration of the db-C4bp protein at its monomer molecular weight, as shown in previous figures and on the left of Figure 9 (showing reduced samples of db-C4bp during purification).

CLAIMS:

1. A method for obtaining a recombinant fusion protein comprising a scaffold of a C-terminal core protein of C4bp alpha chain; said recombinant fusion protein being capable of forming multimers in soluble form in a prokaryotic host cell, the method including the steps of
 - (i) providing a prokaryotic host cell carrying a nucleic acid encoding said recombinant protein operably linked to a promoter functional in said prokaryotic cell;
 - (ii) culturing the host cell under conditions wherein said recombinant protein is expressed; and
 - (iii) recovering the recombinant protein wherein said protein is recovered in multimeric form without performing a scaffold refolding step.
2. A method according to claim 1 wherein the recombinant protein is present at least at a concentration of at least 2 mg/l of cell culture.
3. A method according to claim 1 or claim 2 wherein the host prokaryotic cell is *E. coli*.
4. A method according to claim 3 wherein *E. coli* is selected from strain C41(DE3)[B96070444], C43(DE3)[B96070445] or CO214(DE3)[NCIMB40884], or other strains resistant to the toxicity of overexpressed recombinant proteins.
5. A method according to any one of claims 1 to 4 wherein the recombinant protein comprises the C4bp core protein fused to a heterologous polypeptide.

6. A method according to any one of claims 1 to 6 wherein said heterologous polypeptide is a TNF receptor protein.
7. A method according to any one of the preceding claims wherein said heterologous polypeptide is a BAFF-binding portion of BAFF-R.
8. A method according to any one of claims 1 to 6 wherein said heterologous polypeptide is a thrombopoietin agonist peptide IEGPTLRQWLAARA or somatostatin.
9. An isolated nucleic acid comprising a sequence which encodes a fusion protein of a C-terminal core protein of C4bp alpha chain and BAFF-R.
10. An isolated nucleic acid comprising a sequence which encodes a fusion protein of a C-terminal core protein of C4bp alpha chain and a thrombopoietin agonist peptide IEGPTLRQWLAARA or somatostatin.
11. A prokaryotic expression vector comprising a nucleic acid sequence encoding a fusion protein of a C-terminal core protein of C4bp alpha chain and a heterologous polypeptide operably linked to a promoter functional in prokaryotic cells.
12. A bacterial host cell transformed with the expression vector of claim 11.
13. A protein comprising a C-terminal core protein of C4bp alpha chain fused to BAFF-R.

14. A protein comprising a C-terminal core protein of C4bp alpha chain fused to a thrombopoietin agonist peptide IEGPTLRQWLAARA.

15. A method according to any one of claims 1 to 8 which further comprises formulating said recombinant protein into a composition comprising a pharmaceutically acceptable carrier or diluent.

16. A method for treating a condition in a patient, the condition being associated with raised serum levels of BAFF, said method comprising the steps of administering to a patient a therapeutically effective amount of the protein of claim 14 or nucleic acid of claim 9.

17. A method according to claim 16 wherein the condition is systemic lupus erythematosus.

18. A eukaryotic expression vector comprising a nucleic acid sequence encoding the protein of claim 13 or 14 operably linked to a promoter functional in eukaryotic cells.

19. A eukaryotic host cell transformed with the vector of claim 18.

20. Use of the expression vector of claim 18 in a method of treatment of the human or animal body.

21. A eukaryotic expression vector comprising a nucleic acid sequence encoding a recombinant fusion protein comprising a scaffold of a C-terminal core protein of C4bp alpha chain for the use in the treatment of the human or animal body.

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1/9

HUMAN	5	10	15	20	25	30	35	40	45	50	55	60	65
RABBIT	ET	P	E	G	C	E	Q	V	L	T	G	K	R
RAT	E	V	P	E	G	C	E	Q	V	A	G	R	R
MOUSE	E	V	P	K	D	C	E	H	V	F	A	G	K
BOVINE	E	A	S	E	D	L	K	P	A	L	T	G	N
APOR PIG	E	Y	P	E	G	C	E	Q	V	T	G	R	K
GUINEA PIG	E	Y	P	E	D	C	E	Q	V	H	E	G	K
ZP3 MOUSE	E	V	P	E	E	C	K	Q	V	A	A	G	R

ETPEGCEQVLTGKRLMQCLPNPEDVKMALEVYKLSLEIEQLELQORDSARQSTLDKBL
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 EVPKDCHEVFAGKKLMQCLPNSNDVKMALEVYKLTLEIKQLQLQIDKAKHVDREL
 EASEDLKPALTGNKTMQYVPNSHDVKMALEIYKLTLEVELLQLOIQKBKHTZAH
 EYPGCEQVVTGRKLLQCLSRPEEVKLALEVYKLSLEIEILQTNKLKZAFLLREREKNVTCDFNPE
 EYPEDCEQVHEGKKLMECLPTLEEIKLALYKLSLETNLLLELQIDKKBKAKAKYST
 EVPEECKQVAAGRKLLLECLPNPSPDVKMALEVYKLSLEIEQLEKEKYVKIQEKFSKE
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Figure 1

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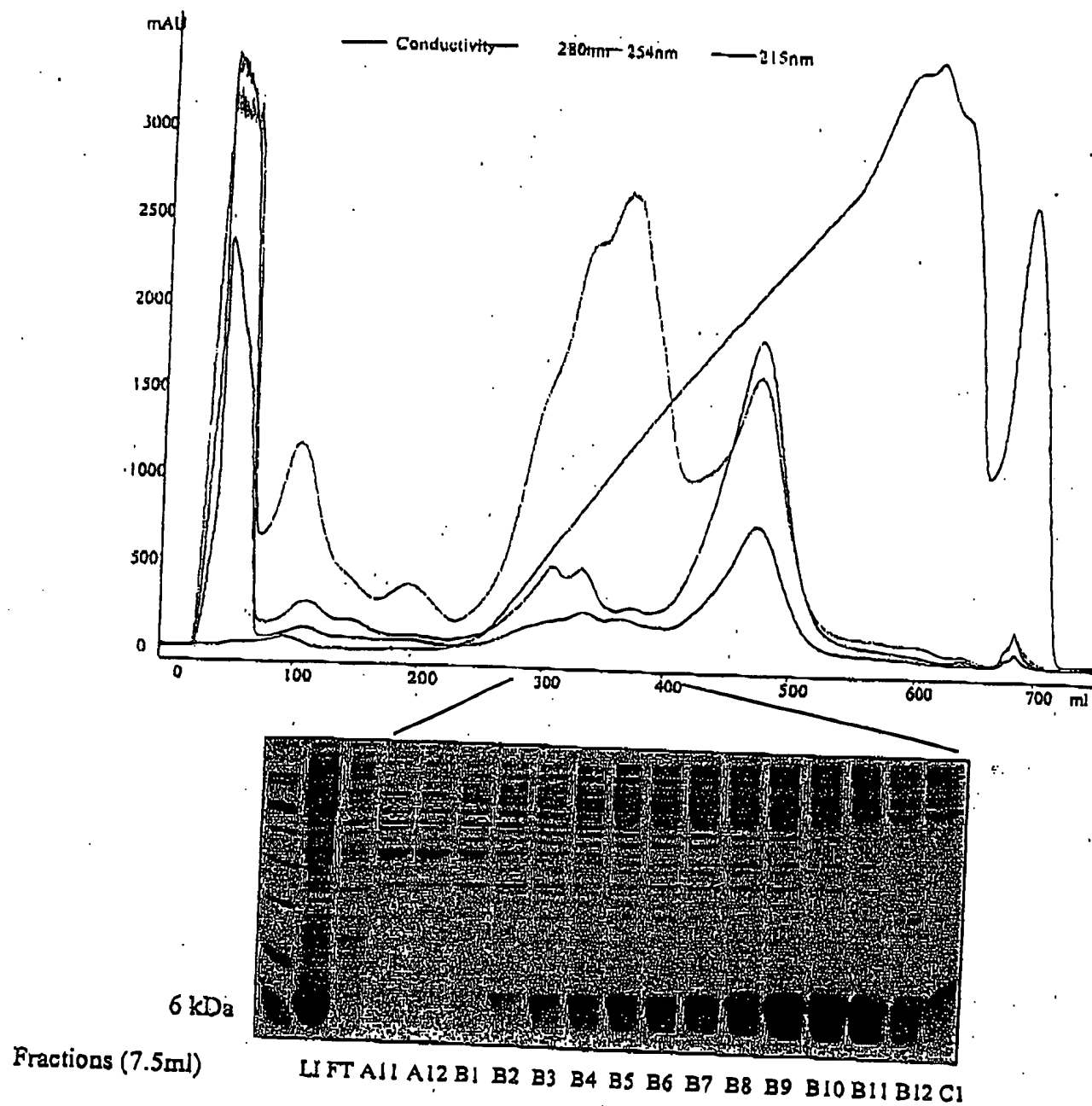


Figure 2

Best Available Copy

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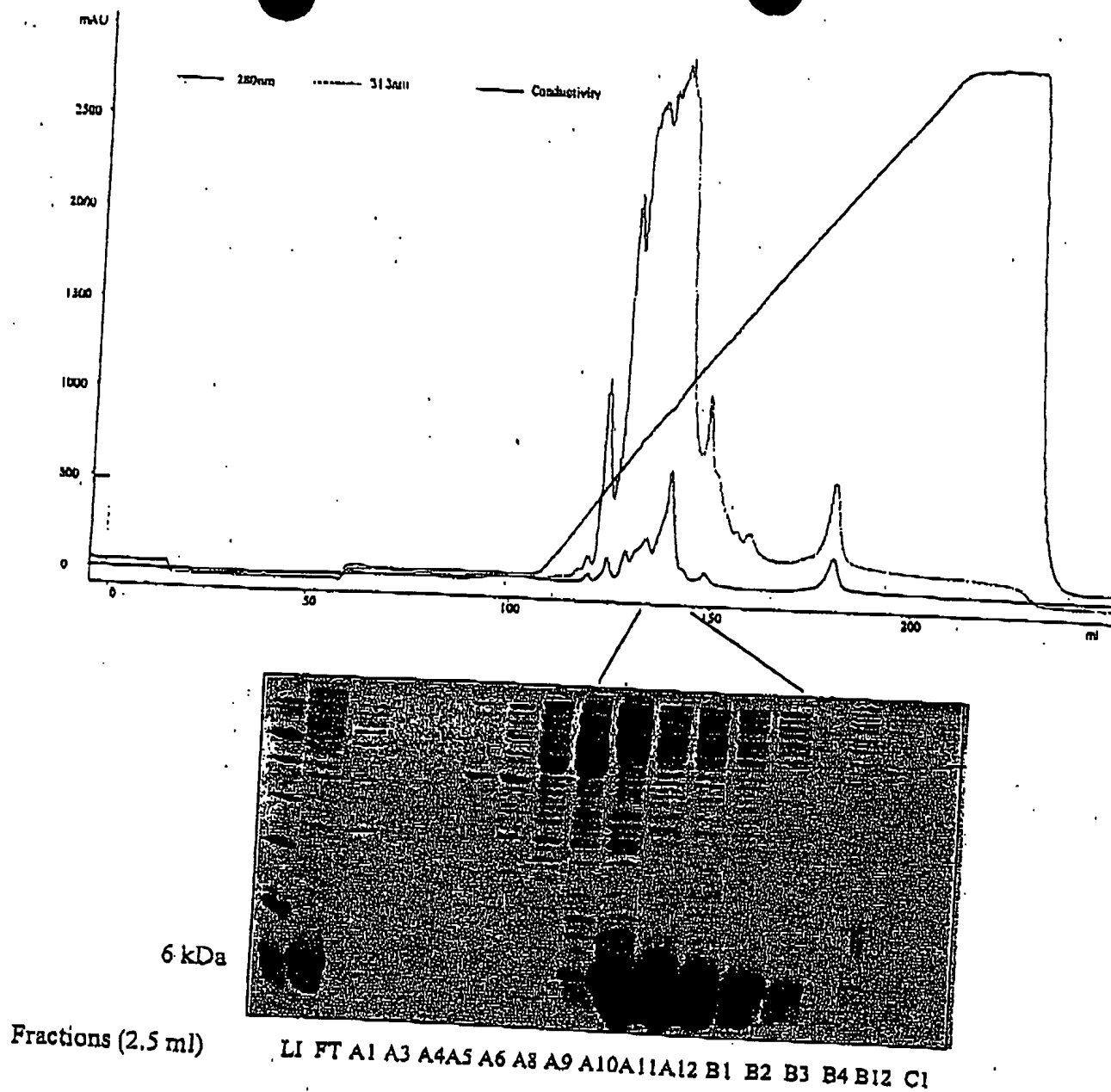
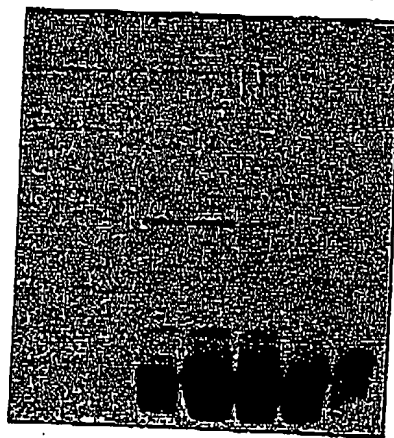
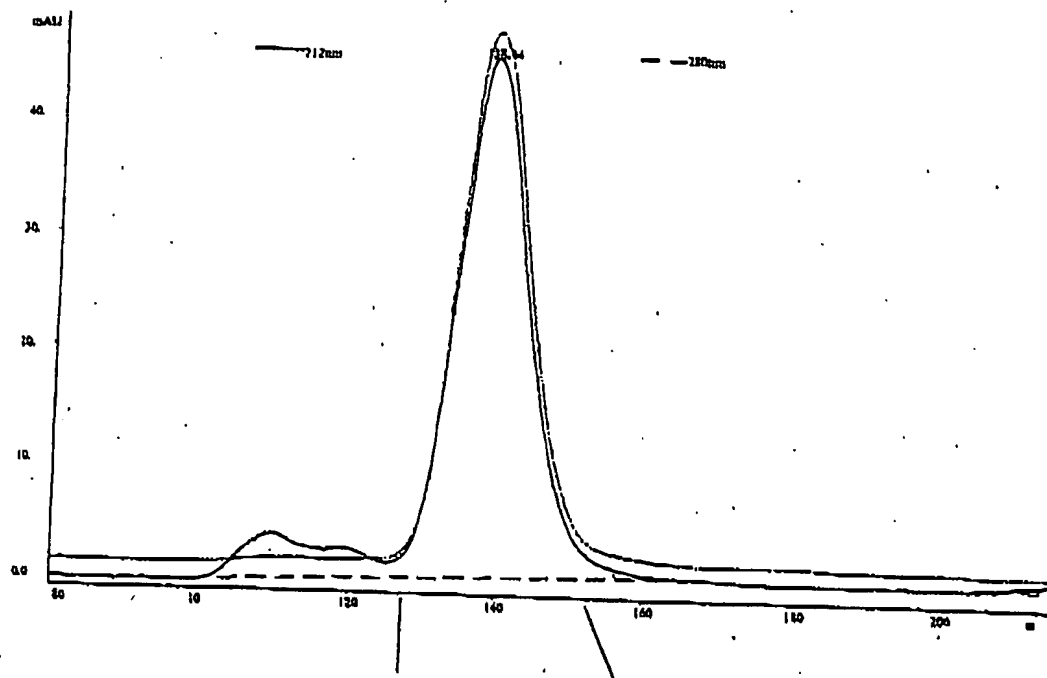


Figure 3

Best Available Copy

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Fractions (5 ml)

A7 A8 A9 A10 A11 A12 B1 B2

Figure 4

Best Available Copy

5/9

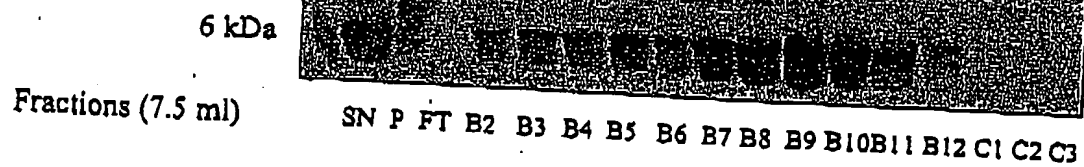
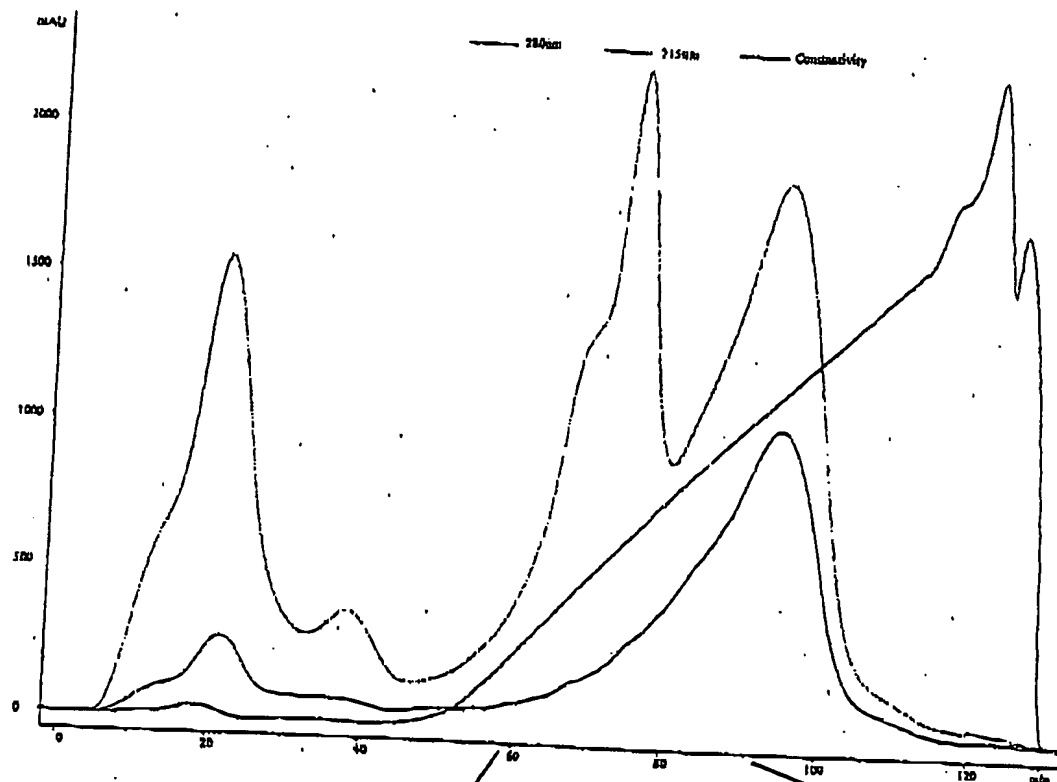


Figure 5

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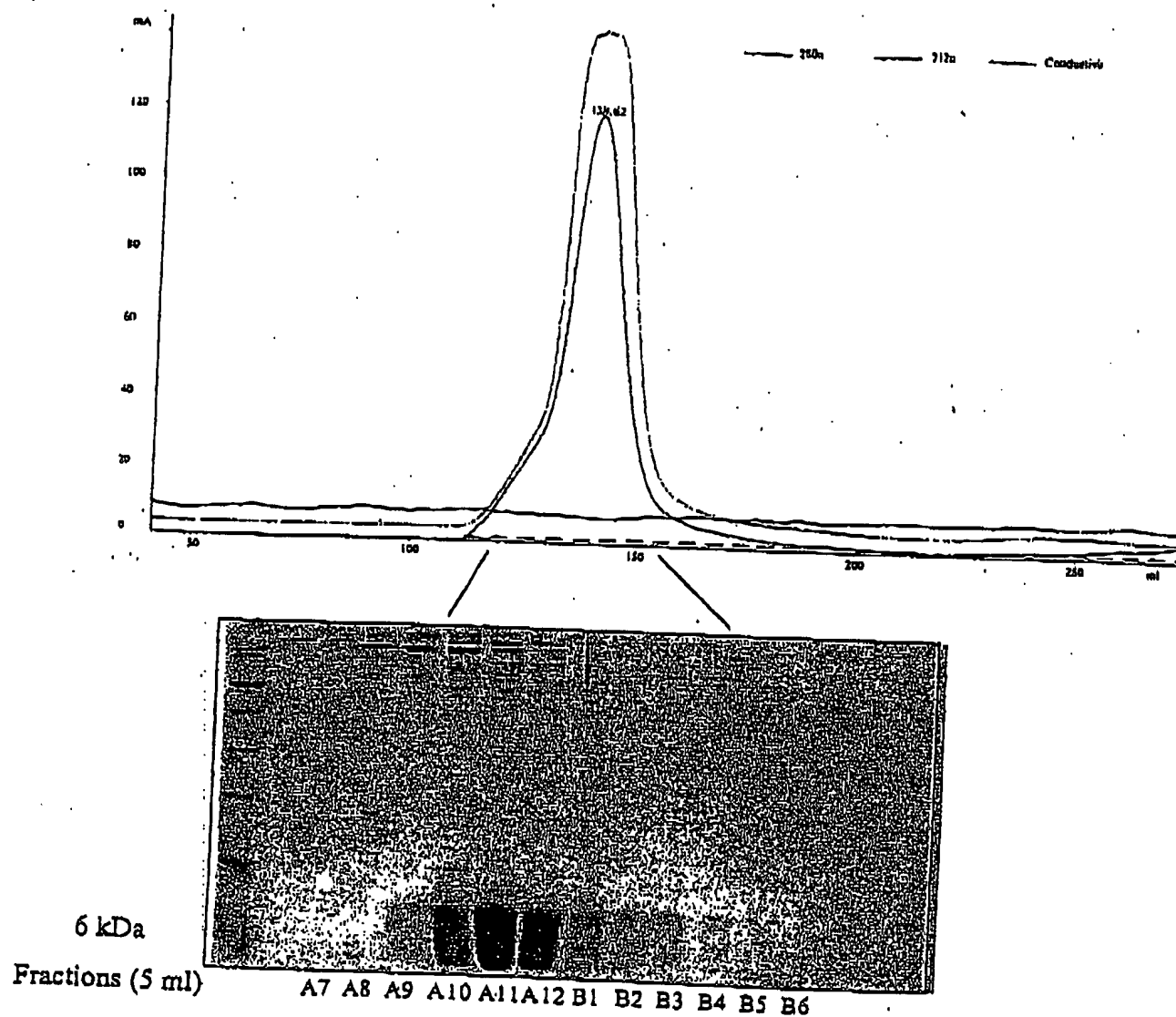


Figure 6

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7/9

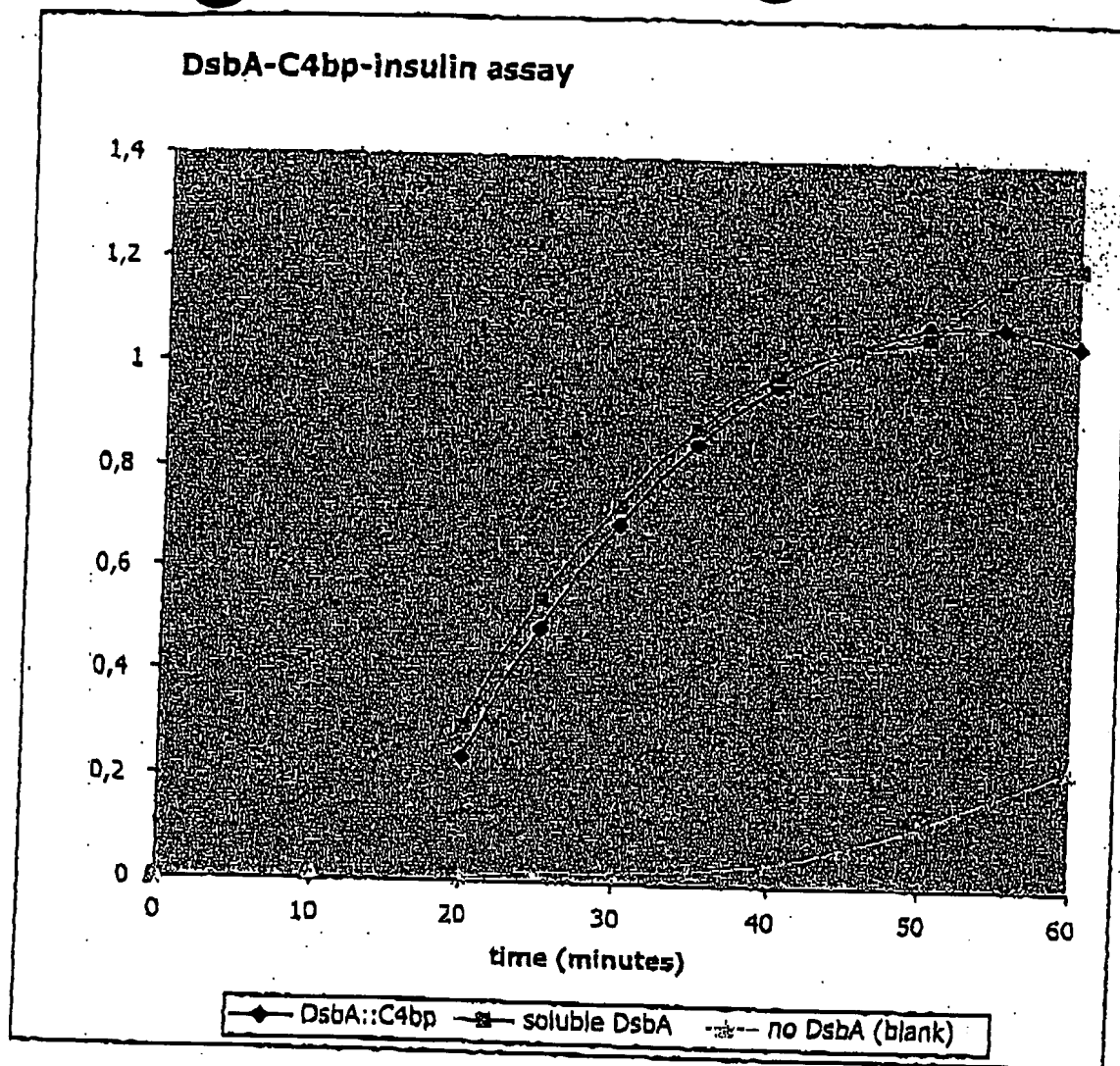


Figure 7

Best Available Copy

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GGATCCgaga ccccggaagg ctgtgaacaa gtgtcacag gcaaaagact catgcagtgt
ctcccaaacc cagaggatgt gaaaatggcc ctggaggtat ataagctgtc tctggaaatt
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taa

Figure 8

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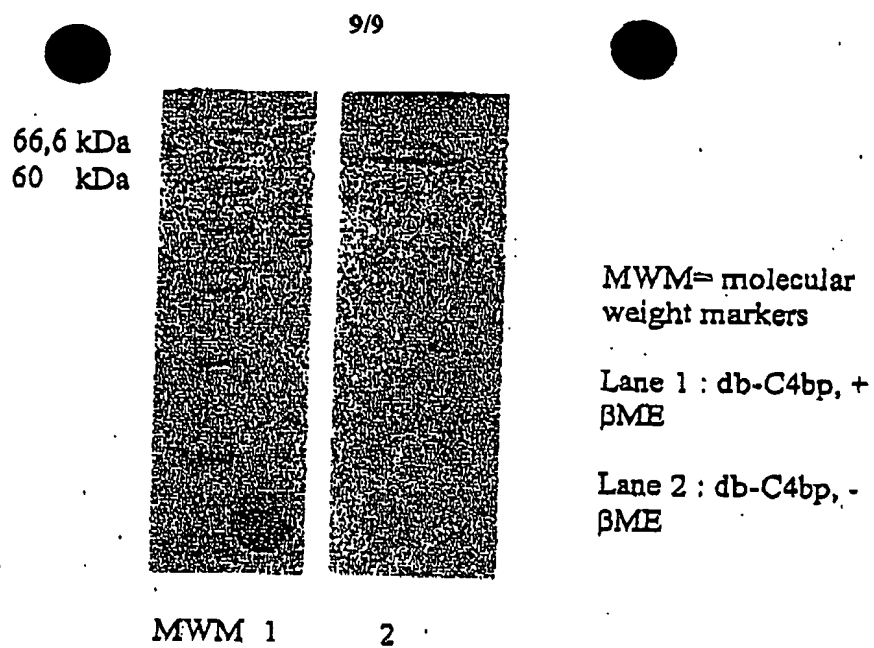


Figure 9

Best Available Copy

1/10

SEQUENCE LISTING

<110> AVIDIS SA

<120> Production of Multimeric Fusion Proteins using a C4bp Scaffold

<130> AHB/FP6155089

<140>

<141>

<150> EP 02292043.3

<151> 2002-08-14

<160> 29

<170> PatentIn Ver. 2.1

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<211> 57

<212> PRT

<213> Homo sapiens

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35 40 45

Arg Gln Ser Thr Leu Asp Lys Glu Leu
50 55

<210> 2

<211> 57

<212> PRT

<213> Oryctolagus cuniculus

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20 25 30

Lys Leu Ser Leu Glu Ile Glu Leu Leu Glu Leu Gln Arg Asp Lys Ala
35 40 45

Arg Lys Ser Ser Val Leu Arg Gln Leu
50 55

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<211> 55

<212> PRT

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<213> Rattus sp.

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Lys Leu Thr Leu Glu Ile Lys Gln Leu Gln Leu Gln Ile Asp Lys Ala
 35 40 45

Lys His Val Asp Arg Glu Leu
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<211> 54

<212> PRT

<213> Mus sp.

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Lys His Thr Glu Ala His
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<211> 67

<212> PRT

<213> Bos sp.

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Gln Cys Leu Ser Arg Pro Glu Glu Val Lys Leu Ala Leu Glu Val Tyr
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Lys Leu Ser Leu Glu Ile Glu Ile Leu Gln Thr Asn Lys Leu Lys Lys
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Asn Pro Glu
 65

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<211> 57

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Lys Lys Ala Lys Ala Lys Tyr Ser Thr
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<212> PRT
<213> Cavia porcellus

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Glu Cys Leu Pro Asn Pro Ser Asp Val Lys Met Ala Leu Glu Val Tyr
20 25 30

Lys Leu Ser Leu Glu Ile Glu Gln Leu Glu Lys Glu Lys Tyr Val Lys
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Ile Gln Glu Lys Phe Ser Lys Glu
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<210> 8
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<213> Mus sp.

<400> 8

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His Cys Leu Ser Ser Pro Glu Asp Val His Arg Ala Leu Lys Val Tyr
20 25 30

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4/10

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C4bp core protein

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1				5					10					15	

Pro	Glu	Asp	Val	Lys	Met	Ala	Leu	Glu	Val	Tyr	Lys	Leu	Ser	Leu	Glu
		20						25					30		

Ile	Glu	Gln	Leu	Glu	Leu	Gln	Arg	Asp	Ser	Ala	Arg	Gln	Ser	Thr	Leu
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Asp	Lys	Glu	Leu
		50	

<210> 10

<211> 57

<212> PRT

<213> Artificial Sequence

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<223> Description of Artificial Sequence: Variant of the
C4bp core protein

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1				5					10					15	

Gln	Cys	Leu	Pro	Asn	Pro	Glu	Asp	Val	Lys	Met	Ala	Leu	Glu	Val	Tyr
		20					25						30		

Lys	Leu	Ser	Leu	Glu	Ile	Lys	Gln	Leu	Glu	Leu	Gln	Arg	Asp	Ser	Ala
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Arg	Gln	Ser	Thr	Leu	Asp	Lys	Glu	Leu
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<210> 11

<211> 52

<212> PRT

<213> Artificial Sequence

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C4bp core protein

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Cys	Glu	Gln	Val	Leu	Thr	Gly	Lys	Arg	Leu	Met	Gln	Cys	Leu	Pro	Asn
1				5					10					15	

Pro	Glu	Asp	Val	Lys	Met	Ala	Leu	Glu	Val	Tyr	Lys	Leu	Ser	Leu	Glu
		20						25					30		

Ile	Lys	Gln	Leu	Glu	Leu	Gln	Arg	Asp	Ser	Ala	Arg	Gln	Ser	Thr	Leu
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

5/10

35

40

45

Asp Lys Glu Leu
50

<210> 12
<211> 57
<212> PRT
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1 5 10 15
Gln Cys Leu Pro Asn Pro Glu Asp Val Lys Met Ala Leu Glu Ile Tyr
20 25 30
Lys Leu Ser Leu Glu Ile Glu Gln Leu Glu Leu Gln Arg Asp Ser Ala
35 40 45
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50 55

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<400> 13
Glu Thr Pro Glu Gly Cys Glu Gln Val Leu Thr Gly Lys Arg Leu Met
1 5 10 15
Gln Cys Leu Pro Asn Pro Glu Asp Val Lys Met Ala Leu Glu Ile Tyr
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<210> 14
<211> 50
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C4bp core protein

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Pro Asn Pro Glu Asp Val Lys Met Ala Leu Glu Ile Tyr Lys Leu Ser
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Thr Leu
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<210> 15

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<400> 15

Glu Thr Pro Glu Gly Ser Glu Gln Val Leu Thr Gly Lys Arg Leu Met
 1 5 10 15

Gln Ser Leu Pro Asn Pro Glu Asp Val Lys Met Ala Leu Glu Val Tyr
 20 25 30

Lys Leu Ser Leu Glu Ile Lys Gln Leu Glu Leu Gln Arg Asp Ser Ala
 35 40 45

Arg Gln Ser Thr Leu Asp Lys Glu Leu
 50 55

<210> 16

<211> 52

<212> PRT

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<223> Description of Artificial Sequence: Variant of the C4bp core protein

<400> 16

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Pro Asn Pro Glu Asp Val Lys Met Ala Leu Glu Ile Tyr Lys Leu Ser
 20 25 30

Leu Glu Ile Glu Gln Leu Glu Leu Gln Arg Asp Ser Ala Arg Gln Ser
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Thr Leu Asp Lys

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50

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linker

<400> 17
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linker

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<210> 20
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linker

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Gly Gly Gly Ser

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20

<210> 21
<211> 14
<212> PRT
<213> Artificial Sequence

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agonist peptide

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Ile Glu Gly Pro Thr Leu Arg Gln Trp Leu Ala Ala Arg Ala
1 5 10

<210> 22
<211> 9
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peptide sequence

<400> 22
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1 5

<210> 23
<211> 31
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<210> 24
<211> 37
<212> DNA
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<400> 24
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37

<210> 25
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<213> Artificial Sequence

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encoding a 6xHistidine tag

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<210> 26

<211> 74

<212> PRT

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<223> Description of Artificial Sequence: Amino acid
sequence produced by plasmid pAVD 93

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1 5 10 15Ser Glu Thr Pro Glu Gly Cys Glu Gln Val Leu Thr Gly Lys Arg Leu
20 25 30Met Gln Cys Leu Pro Asn Pro Glu Asp Val Lys Met Ala Leu Glu Val
35 40 45Tyr Lys Leu Ser Leu Glu Ile Glu Gln Leu Glu Leu Gln Arg Asp Ser
50 55 60Ala Arg Gln Ser Thr Leu Asp Lys Glu Leu
65 70

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38

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<211> 48

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48

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<210> 29
<211> 303
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<220>
<223> Description of Artificial Sequence: Promoter and
C4bp coding region in pAVD77

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ggatccgaga ccccggaagg ctgtgaacaa gtgctcacag gcaaaagact catgcagtgt 180
ctcccaaacc cagaggatgt gaaaatggcc ctggagggtat ataagctgtc tctggaaatt 240
gaacaactgg aactacagag agacagcgca agacaatcca ctttggataa agaactataa 300
taa 303

INTERNATIONAL SEARCH REPORT

Ints. Application No

PCT/IB2004/002717

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K19/00 A61K39/385 A61P33/06 A61P31/16 A61K39/395
A61K39/42 C07K16/20 C07K16/10 C12N15/70 C12N15/79
C12N1/21 C12N5/10 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ, Sequence Search, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 91/11461 A (BIOGEN, INC.) 8 August 1991 (1991-08-08) cited in the application page 7, line 10 - line 16. page 9, line 32 - page 10, line 16 figure 9 examples IV-VII	1-7,9-15
Y	----- -/-	16

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

Y later document published after the international filing date of priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

A document member of the same patent family

Date of the actual completion of the international search

19 April 2005

Date of making of the international search report

18-07-2005

Name and mailing address of the ISA

European Patent Office, P.O. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 cpo nl.
Fax: (+31-70) 340-3016

Authorized officer

Noord, F

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB2004/002717

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	E. SHINYA ET AL.: "In-vivo delivery of therapeutic proteins by genetically-modified cells: comparison of organoids and human serum albumin alginate-coated beads." BIOMEDICINE AND PHARMACOTHERAPY, vol. 53, December 1999 (1999-12), pages 471-483, XP002231127 Paris, France cited in the application abstract page 481, left-hand column -----	1,2,4,5, 9,10
X	D. CHRISTIANSEN ET AL.: "Octamerization enables soluble CD46 receptor to neutralize measles virus in vitro and in vivo." JOURNAL OF VIROLOGY, vol. 74, no. 10, May 2000 (2000-05), pages 4672-4678, XP002231126 USA abstract page 4677, left-hand column, line 25 - line 38 -----	1,2,4,5, 9,10
X	M. TONYE LIBYH ET AL.: "A recombinant human scFv anti-Rh(D) antibody with multiple valences using a C-terminal fragment of C4-binding protein." BLOOD, vol. 90, no. 10, 15 November 1997 (1997-11-15), pages 3978-3983, XP002231124 NEW YORK, NY, USA abstract figure 1 -----	1,2,4-6, 9
Y	US 5 219 987 A (VERDINI ET AL.) 15 June 1993 (1993-06-15) the whole document -----	16
P,X	WO 2004/020639 A (AVIDIS SA) 11 March 2004 (2004-03-11) examples 1-6 figure 1 page 25, line 14 - line 15 -----	1-7,9-16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/182004/002717**Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos. because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6 (all partially), 7 (completely), 9, 10 (both partially)
11-16 (all completely)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

International Application No. PCT/182004/002717

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-6 (all partially), 7 (completely), 9, 10 (both partially), 11-16 (all completely)

Product comprising a C4bp core protein and a monomeric antigen, wherein said monomeric antigen is a monomeric antigenic fragment of a Plasmodium merozoite surface protein 1. Composition comprising said product. Method of immunotherapy of malaria using said product. Use of said product for the manufacture of a medicament for the treatment or prevention of malaria. Method of producing antibodies against a Plasmodium parasite using said product.

2. claims: 1-6 (partially), 8 (completely), 9, 10 (both partially), 20-25 (all completely)

Product comprising a C4bp core protein and a monomeric antigen, wherein said monomeric antigen is a monomeric antigenic fragment of influenza virus hemagglutinin protein or the influenza M2e peptide. Composition comprising said product. Method of immunotherapy of influenza using said product. Use of said product for the manufacture of a medicament for the treatment or prevention of influenza. Method of producing antibodies against influenza using said product.

3. claims: 17 (partially), 18, 19 (both completely)

Method of passive immunization against a disease of a subject, said method comprising administering to said subject an immune serum containing antibodies, and wherein said disease is malaria. Immune serum obtained by a claimed method for use in a method of immunotherapy of malaria in a human subject.

4. claims: 17 (partially), 26, 27 (both completely)

Method of passive immunization against a disease of a subject, said method comprising administering to said subject an immune serum containing antibodies, and wherein said disease is influenza. Immune serum obtained by a claimed method for use in a method of immunotherapy of influenza in a human subject.

5. claims: 28 (completely), 30, 31 (both partially)

Method of making a product comprising a C4bp core protein and a non-polypeptide monomeric antigen.

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International Application No. PCT/IB2004/002717

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

6. claims: 29 (completely), 30, 31 (both partially)

Method of making a product comprising a fusion of a C4bp core protein and a polypeptide monomeric antigen.

7. claim: 32 (completely)

Method for increasing the immunogenicity of a monomeric antigen, said method comprising joining said antigen to a C4bp core protein.

8. claims: 33-38 (all completely)

Expression vector comprising a nucleic acid sequence encoding a fusion protein of a C4bp core protein and a polypeptide monomeric antigen, operably linked to a promoter functional in host cell. Bacterial and eukaryotic host cells transformed with said vector. Use of said vector in a method of treatment of the human or animal body.

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INTERNATIONAL SEARCH REPORT

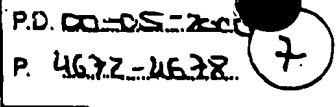
International Application No
PCT/IB2004/002717

Parent document cited in search report		Publication date	Patent family member(s)	Publication date
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Octamerization Enables Soluble CD46 Receptor To Neutralize Measles Virus In Vitro and In Vivo

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A chimeric fusion protein encompassing the CD46 ectodomain linked to the C-terminal part of the C4b binding protein (C4bp) α chain (sCD46-C4bp α) was produced in eukaryotic cells. This protein, secreted as a disulfide-linked homo-octamer, was recognized by a panel of anti-CD46 antibodies with varying avidities. Unlike monomeric sCD46, the octameric sCD46-C4bp α protein was devoid of complement regulatory activity. However, sCD46-C4bp α was able to bind to the measles virus hemagglutinin protein expressed on murine cells with a higher avidity than soluble monomeric sCD46. Moreover, the octameric sCD46-C4bp α protein was significantly more efficient than monomeric sCD46 in inhibiting virus binding to CD46, in blocking virus induced cell-cell fusion, and in neutralizing measles virus in vitro. In addition, the octameric sCD46-C4bp α protein, but not the monomeric sCD46, fully protected CD46 transgenic mice against a lethal intracranial measles virus challenge.

Control of virus infection is currently a major challenge. The identification of cellular receptors used by viruses to enter their host cells has allowed several groups to investigate the potential antiviral properties of recombinant soluble receptors. Whereas recombinant soluble CD4 and Tva exhibited high neutralizing properties, at least in vitro, against human immunodeficiency virus (13, 19, 27, 46) and subgroup A avian sarcoma and leukosis viruses (5, 11), respectively, the anti-measles virus (anti-MV) activity of recombinant soluble monomeric CD46 (sCD46) against MV was very poor (16, 45). Since MV virus binding and fusion to cells likely involves several CD46 receptor molecules (7), we hypothesized that a multimeric form of soluble CD46 could have more potent antiviral activity.

Measles virus, a member of the order *Mononegavirales*, is responsible for an acute human pulmonary disease with high morbidity and mortality, killing over 1 million young children every year, mainly in developing countries. Infection is associated with a profound but transient cellular immunodepression. In rare cases, MV can induce lethal neuropathological diseases, acute encephalopathy, measles inclusion bodies encephalitis, or subacute sclerosing panencephalitis. MV attenuated by growth in chicken embryonic fibroblasts is currently used as an effective but limited vaccine because of its inefficiency in children less than 9 months old.

Human CD46 (or membrane cofactor protein), which is expressed on all cells except erythrocytes, is used as a cellular receptor by at least a subgroup of laboratory and wild-type MV strains (17, 38; see reference 22 for a review) through the interaction of its ectodomain with that of the MV envelope glycoprotein hemagglutinin (H) (16). This MV H-CD46 interaction induces a multimolecular scaffold in which the MV fusion glycoprotein (F) initiates the fusion between the MV

envelope and the plasma cell membrane at a neutral pH (7). These properties explain the occurrence of cell-cell fusion observed after MV infection. CD46 is a transmembrane glycoprotein that belongs to the regulators of complement activation gene family. The dominant structural units of CD46 are the four short consensus repeat (SCR) domains of 60 to 64 amino acids that are responsible for complement binding and regulatory functions. Structurally, the N-terminal four SCRs of CD46 precede a heavily glycosylated serine-threonine-proline (STP)-rich domain, a transmembrane domain, and one of two alternative cytoplasmic tails. CD46 protects all cells but erythrocytes from complement activation by acting as a cofactor for the factor I serine protease, which cleaves C3b (see reference 30 for a review), and also prevents the alternative pathway amplification loop of C3b deposition on the cell surface (15). SCRs II, III, and IV are required for this cofactor activity, with SCRs III and IV being mainly involved in the binding to C3b (1).

The H binding site on CD46 has been mapped to the first two N-terminal SCR domains (7, 28, 34, 40). Modeling of CD46 SCR domains I and II (37), which was recently proved to be largely correct following X-ray diffraction analysis of CD46 SCR I and II crystals (8), together with H, MV, and antibody binding studies on site-directed mutated CD46 protein (6, 26, 32), indicated that the H protein interacts on one face extending from the top of SCR I to the bottom of SCR II. Although dispensable for MV binding, the underlying SCR III and IV domains optimize this interaction (14), with SCR IV playing a major role (9). The STP regions are not directly involved in CD46-mediated MV entry (21, 33).

The poor antiviral activity of a recombinant soluble form of CD46 (16, 45) led to the design of an oligomeric form of the receptor. This was based on C4b binding protein (C4bp), another complement regulatory molecule. This molecule is a multimer associating seven α chains, each consisting of eight SCR domains linked to a C-terminal oligomerization peptide, and one β chain composed of three SCR domains linked to an

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oligomerization peptide. When adsorbed to thin carbon films and examined under electron microscopy, C4bp has a spider-like structure (12, 47). A fusion protein between the four CD46 SCR domains, STP B region, and the oligomerization site of the C4bp α chain was generated and tested for its MV-neutralizing properties.

MATERIALS AND METHODS

Cloning procedure and isolation of cell lines producing sCD46-C4bp fusion protein. A cDNA encoding for the signal peptide and the first 269 amino acids of CD46, which encompasses the four SCR domains and the STP B region, was fused to a cDNA encoding the amino-acid 57-C-terminal sequence of the C4bp α chain, which encompasses the C4bp multimerization domain. The cDNA encoding the CD46-C4bp chimeric protein was subcloned under the simian virus 40 promoter in the pKC3 eukaryotic vector (29). After cotransfection with the pMANNeo plasmid (encoding for neomycin resistance) into CHO cells, stable clones secreting the CD46-C4bp protein were isolated and amplified as published (29). The cDNA encoding the sCD46-C4bp protein was also subcloned under the cytomegalovirus promoter into the APEX3 vector (10), which was used to derive human 293EBNA cells expressing the chimeric protein. The secretion of the chimeric protein was determined using a dot blot assay with MCI20.6 anti-CD46 antibody and anti-mouse immunoglobulin (Ig)-alkaline phosphatase conjugate as previously detailed (20). Throughout all the analyses, an immunopurified recombinant monomeric sCD46 previously described (10) was used for comparative studies.

Purification and biochemical characterization of sCD46-C4bp chimeric protein. The recombinant sCD46-C4bp protein was immunopurified from cell supernatant using the anti-CD46 monoclonal antibody (MAb) B4.3 immobilized on activated Sepharose 4B (Pharmacia) according to the manufacturer's instructions and was eluted with 0.1 M HCl-glycine buffer (pH 2.8). Alternatively, the sCD46-C4bp protein was produced in serum-free medium supplemented with the plant-derived growth factor ProLifin (Biomedica), concentrated on 100-kDa exclusion membrane, and purified by exclusion chromatography on Sepharose 200 (Pharmacia). Metabolic labeling with Tran³⁵S-label (NEN) for 30 min followed by a chase of 2 h and immunoprecipitation of cell extract and cell supernatant using J4-48 antibodies and protein G-Sepharose beads were performed according to published procedures (20, 38).

Matrix-assisted laser desorption mass spectrometry (MALDI MS). After dialysis and concentration under vacuum, the sample was dissolved in 0.1% trifluoroacetic acid and mixed with matrix (saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid in water:acetonitrile [2:1, vol/vol]). One microliter of the mixture with a matrix-to-sample ratio of ca. 10,000 was deposited on a thin layer of matrix crystals prepared on the target. After drying in air at ambient temperature, resulting crystals were analyzed in the mass spectrometer (Bruker Biflex; Bremen, Germany). External calibration was made with carboxylic anhydride (29,024 kDa) or monomeric and dimeric bovine serum albumin (66,430 and 132,858 kDa). Spectra were acquired in linear mode (turbo mode) at an acceleration voltage of 28 kV.

Enzyme-linked immunosorbent assay procedure. The reactivity of sCD46-C4bp with anti-CD46 antibodies was tested in an antibody binding competition assay on immobilized recombinant sCD46 revealed by a phosphatase alkaline-anti-mouse Ig conjugate as previously detailed (5, 16). The apparent avidity of antibodies was determined from the representation of Lineweaver and Burk, i.e., 1/bound antibody as a function of 1/antibody concentration (6).

C3b deposition. Human C3b deposition on CHO and CHO-CD46 cells following activation of the alternative complement pathway was performed essentially as recently described (15). Briefly, CHO cells were incubated with human serum diluted 1:3 in the presence of 20 mM MgCl₂, 100 mM EGTA, and serial dilutions of either sCD46-C4bp or sCD46 for 1 h at 37°C. After being washed, the cells were immunolabeled using anti-C3b(C3c) WMI antibody and anti-mouse IgG-phycoerythrin conjugate. The level of C3b was measured by flow cytometry.

Cytofluorescence assays. The binding of sCD46-C4bp to transmembrane MV H protein was tested after incubation of serially diluted protein with 2 × 10⁵ L cells expressing MV H glycoprotein in a round-bottom 96-well microtiter plate (30 min at 20°C). Following washing, cells were incubated with an appropriate dilution of the anti-CD46 G824 antibody. Phycoerythrin-anti-mouse IgG (heavy- and light-chain) conjugate was added following additional washing and cytofluorescence analysis performed as detailed previously (16). The results were expressed in mean fluorescence values and used to calculate the apparent avidity of sCD46-C4bp from the representation of Lineweaver and Burk.

Virus binding assay. The assay was performed essentially as described (26). Serial dilutions of the recombinant sCD46 material were incubated with purified MV (Hallé strain) for 1 h at 20°C, and the mixture was added to CHO-CD46 cells. MV binding was measured after immunolabeling and flow cytometry.

Cell fusion. A quantitative fusion assay based on the conditional expression of β -galactosidase (β -Gal) under the control of the T7 polymerase promoter was used. Briefly, the first HeLa cell partner was infected with MV (Hallé strain) at a multiplicity of infection (MOI) of 2 (or 8 h at 37°C. After 7 h of incubation a recombinant vaccinia virus encoding the T7-DNA-dependent RNA polymerase

(WT) (at an MOI of 1) was added; at the end of the 8-h period the cells were washed once and cultured for an additional 16 h in the presence of a fusion peptide inhibitor, *N*- α -Phe-Phe-Gly, to prevent ongoing cell fusion and cell death. A second cell partner was infected with a recombinant vaccinia virus encoding the T7-driven β -Gal cDNA (vCB21R-lacZ) (at an MOI of 1) (3) for 1 h, washed once, and then incubated for an additional 16 h at 37°C. MV- and WT-infected cells were washed three times to eliminate the *N*- α -Phe-Phe-Gly peptide and resuspended in culture medium supplemented with 40 μ M AraC to stop the replication of vaccinia virus and reduce nonspecific induction of β -Gal activity (39). A total of 10⁵ cells were coinoculated with a serial dilution of either sCD46, sCD46-C4bp, monoclonal anti-MV-H 48d6, anti-MV-P Y503, or anti-human C3b WMI antibody for 30 min at 4°C prior to the addition of 10⁵ vCB21R-lacZ infected cells. After 6 h of incubation at 37°C, the cells were lysed and the β -Gal activity was determined by colorimetry using *p*-nitrophenyl- β -D-galactopyranoside substrate (Sigma).

In vitro neutralization assay. Two different methods were used. In the first one, serial dilutions of sCD46, sCD46-C4bp, or bovine serum albumin were incubated for 30 min at 37°C with 100 PFU of MV (Edmonston strain) (American Tissue Culture Collection) in tissue culture medium supplemented with 2% fetal calf serum. The mixture was then layered onto a Vero cell monolayer and incubated for 4 days. Cells were fixed in 10% formalin and stained with methyl blue, and the number of PFU was counted. The second method was devised to test the reversibility of the virus neutralization. Briefly, 10⁵, 10⁴, 10³, and 10² 50% tissue culture infective doses (TCID₅₀) of MV (Hallé strain) was incubated with 300 μ g of sCD46-C4bp, WMI anti-C3b, 48d6 anti-MV-H, or Y503 anti-MV-P antibodies per ml in a final volume of 30 μ l for 1 h at 37°C. After the addition of 270 μ l of culture medium, serial dilutions (1:3) of the mixture were made in 96-well microtiter plates, with each dilution being equally aliquoted into eight wells. Vero cells (10⁵ in 200 μ l) were added to each well, and the microplates were incubated for 10 days at 37°C. Under these conditions, the final concentration of the inhibitor during the Vero cell infection step was inferior or equal to 10 aM at most. Infectious virus was quantified using the TCID₅₀ procedure.

In vivo neutralization assay. Transgenic mice ubiquitously expressing the human CD46 protein (line MCP-7) have been described previously (25). These mice were shown to be highly sensitive to intraperitoneal infection by MV (18). Before infection, phosphate-buffered saline (PBS), sCD46, or sCD46-C4bp was mixed with either MV (Edmonston strain) or canine distemper virus (CDV) (Ondara strain) and incubated for 15 min at 37°C. This mixture (30 μ l) was then used to intraperitoneally inoculate 2- to 3-day-old suckling CD46 transgenic mice. Animals were observed for clinical symptoms and death daily for 10 weeks.

RESULTS

CD46-C4bp protein is secreted as a homo-octamer. After transfection with CD46-C4bp, pKC3 or APEX-CD46-C4bp eukaryotic vectors and selection in the presence of the appropriate antibiotics, several clones of CHO and 293EBNA cells were found to secrete a protein which reacted with the MCI20.6 anti-CD46 MAb in a dot blot assay. One of the CHO cell clones, 2B5, was metabolically radiolabeled for 30 min and then subjected to a chase of 2 h. From the cell extract, the anti-CD46 MAb J4-48 specifically immunoprecipitated a protein which resolved after polyacrylamide gel electrophoresis into a doublet of ~50 kDa and a single band of ~250 kDa under reducing and nonreducing conditions, respectively (Fig. 1a, lanes 3 and 7). From the cell supernatant, the immunoprecipitated material resolved into broad bands of ~65 and ~320 kDa under reducing and nonreducing conditions, respectively (Fig. 1a, lanes 4 and 8). In addition, a band with a mass of >200 kDa, which could correspond to unreduced sCD46-C4bp, was also detected under reducing conditions (Fig. 1a, lane 4). The size increase of the secreted material as well as the broadening of the bands probably reflects heterogeneous glycosylation, which is typically observed with CD46 (30, 38).

Sufficient material was immunopurified on an B4.3 MAB column for MALDI MS analysis. The spectrum resolved into 12 peaks labeled as follows: a, 19,086 Da; b, 37,963 Da; c, 42,772 Da; d, 80,735 Da; e, 100,232 Da; f, 120,899 Da; g, 139,233 Da; h, 159,945 Da; i, 199,079 Da; j, 238,363 Da; k, 277,362 Da; and l, 317,421 Da. This series was completely different from those resulting from usual multiply-charged ions or from ion signals corresponding to molecular clusters (dimer

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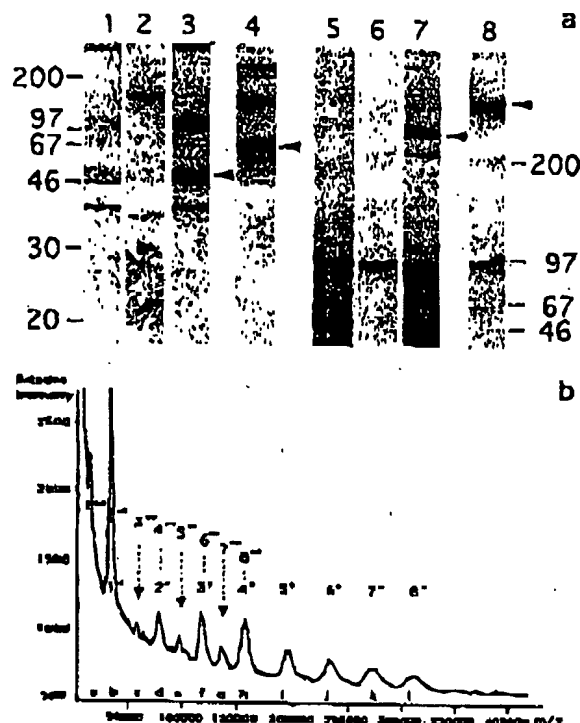


FIG. 1 (a) Polyacrylamide gel electrophoresis autoradiogram of metabolically ^{35}S -labeled CD46-C4bp α immunoprecipitated using 14-6 anti-CD46 antibody under reducing (lanes 1 to 4) and nonreducing (lanes 5 to 8) conditions of cell extracts from CHO (lanes 1 and 5), CHO-CD46-C4bp α cells (lanes 3 and 7), and from supernatant of CHO (lanes 2 and 6) or CHO-CD46-C4bp α (lanes 4 and 8) cells. Major bands specifically immunoprecipitated are indicated by arrows. A prominent nonspecific band around 100 kDa was also observed. (b) MALDI MS of sCD46-C4bp α protein. Peaks labeled b, d, f, h, i, j, k, and l are related to the singly charged ion series, and peaks a through g are related to the doubly charged ion series. Peaks b, d, f, and h correspond to ions belonging to both singly and doubly charged series. Ion species of the singly and doubly charged series contributing to each peak are indicated above the curve. The mass errors range between 0.01 (peak b) and 1.7% (peak i).

[2M + H] $^{+}$, trimer [3M + H] $^{+}$, etc.) which may appear in MALDI MS at high analyte concentration. Indeed, as shown in Fig. 1b, the peak intensities indicate that the twelve peaks actually correspond to eight singly charged ions (peaks b, d, f, h, i, j, k, and l) and eight doubly charged ions (peaks a to g).

The ion nomenclature is based on the number (one to eight) of ~40-kDa subunits and the number of protons bound to the fragment. Four of these peaks (b, d, f, and h) are obviously heterogeneous and reflect the occurrence of one singly and one doubly charged ion. For example, the 4 $^{+}$ and 8 $^{2+}$ ions contribute to the intensity of peak h. The molecular masses (MMs) and intensities of the 16 ion species suggest that they are produced by eight fragments, or subunits, of a native molecule differing by an incremental MM of approximately 40 kDa. Furthermore, the spectrum shows that, as expected, (i) the MM of ions producing peaks b, d, f, and h are exactly those that can be expected for the 8 $^{2+}$ and 4 $^{+}$, 6 $^{2+}$ and 3 $^{+}$, 4 $^{+}$ and 2 $^{+}$, and 2 $^{+}$ and 1 $^{+}$ ions, respectively, of a 317-kDa whole molecule; (ii) peaks a, c, e, and g, which are presumed to be produced by a single ion type, have much lower intensities than their neighbors (peaks b, d, f, and h), corresponding to a mixture of singly and doubly charged ions; (iii) peaks i, j, k, and l exhibit decreasing intensities as expected for a series of ions with increasing masses; and (iv) peak i, the peak with the highest MM of the series, has a MM 16 times greater than peak a and 8 times greater than peak b. The expected nature and the location in the spectrum of the 16 singly and doubly charged ions is given in Fig. 1.

We conclude that the peak series correspond to the monomer, dimer, trimer, tetramer, pentamer, hexamer, heptamer, and octamer of a subunit with a MM of 37,963 Da. Mass values may be slightly underestimated because of possible laser beam-induced carbohydrate loss during MALDI analysis of glycoprotein samples. Thus, the CD46-C4bp α chimerical protein was secreted as homo-octamers linked by disulfide bonds.

Reactivity of sCD46-C4bp α protein with anti-CD46 antibodies. The sCD46-C4bp α protein was found to react with a panel of anti-CD46 MAbs directed against CD46 SCR-I, SCR-II, SCR-III, or SCR-IV domains (Table 1). However, when compared to monomeric sCD46, the octameric sCD46-C4bp α exhibited an increase in avidity to E4.3 (70-fold), MC120.6 (3-fold), and GB24 (2-fold) antibodies, whereas a decrease in avidity to M75 antibody (11-fold) was observed. Similar avidity values were observed with other anti-CD46 MAbs, TRA2.10 and 10.88. Taken together this indicates that, upon multimerization, some minor conformational changes of SCR domains of CD46 occurs. This pattern of antibody reactivity was also different from that observed with the natural transmembrane CD46, which exhibits the highest avidity for all antibodies tested.

sCD46-C4bp α has no complement regulatory activity. The sCD46-C4bp α was mixed with human serum and incubated with CHO cells to determine its ability to prevent the amplification loop of C3b deposition of the alternative complement

TABLE 1. Apparent avidity (K_D) of anti-CD46 antibodies for transmembrane CD46 (tnCD46) expressed on CHO cells, as determined by flow cytometry, and for monomeric sCD46 or octameric sCD46-C4bp α proteins, as determined by competition enzyme-linked immunosorbent assay on immobilized sCD46 α

Antibody	Binding site on CD46	K_D (nM) of anti-CD46 antibodies for:			Inhibitory activity against:	
		tnCD46	sCD46	sCD46-C4bp α	Cofactor activity	MV sH binding
E4.3	SCR-I	0.4	47.2	0.7	-	+/-
MC120.6	SCR-I	0.3	6.1	2.1	-	+
TRA2.10	SCR-I	0.4	36.0	19.6	-	+
M75	SCR-II	0.2	0.3	3.3	+++	+++
GB24	SCR-III and IV	0.3	4.6	2.0	+++	-
10.88	SCR-III and IV	0.3	1.9	2.3	?	-

* The data on the inhibitory activities against complement cofactor activity (underlined) and MV sH binding (in boldface type) are from references 1, 13, and 44 and reference 6, respectively. Symbols: -, none; +, moderate; +/-, low; +++, high; ?, not known.

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sCD46 RECEPTOR NEUTRALIZES MV IN VITRO AND IN VIVO 4675

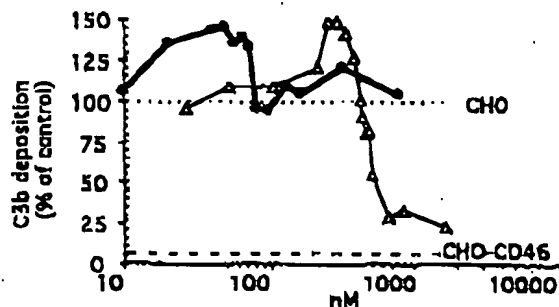


FIG. 2. C3b deposition after alternative complement activation of human serum on CHO cells in the presence of octameric sCD46-C4bpa (circles) or monomeric sCD46 (triangles) proteins. The results are expressed as a percentage of the C3b deposition level observed on CHO cells in the absence of inhibitor. The level of deposition of C3b on CHO-CD46 cells is indicated by the bottom horizontal dotted line. The results are cumulative data from four different experiments.

pathway (Fig. 2). The sCD46-C4bpa protein was unable to decrease the level of the C3b deposition on CHO cells even at the highest concentration tested (250 µg/ml or 780 nM, equivalent to 6240 nM of monovalent CD46). In contrast, and in agreement with previous work (10), monomeric sCD46 at concentrations of >420 nM (i.e., 25 µg/ml) displayed cofactor activity. At concentrations of >1,000 nM, monomeric sCD46 was almost as efficient as transmembrane CD46, which completely prevented the amplification loop of the C3b deposition, leaving only around 5% of the C3b deposition, corresponding to the primary tick-over phase (Fig. 3 [bottom dotted line]) (15). In addition, with intermediate concentrations of both of the sCD46-C4bpa (10 to 80 nM, equivalent to 80 to 640 nM monovalent CD46) and sCD46 (150 to 400 nM) proteins, the amount of C3b deposition was higher than on untreated CHO cells (Fig. 3 [see values above the upper dotted line]). This enhancement was not observed when the complement activation was performed on CHO-CD46 cells (not shown).

sCD46-C4bpa protein can bind to MV H. Both the octameric sCD46-C4bpa and monomeric sCD46 were able to bind to L cells expressing the MV H protein but not to the parental murine L cells (not shown). sCD46-C4bpa exhibited a 2.5-fold-higher apparent avidity (48 nM, equivalent to 384 nM monovalent CD46) towards H protein than sCD46 (119 nM).

sCD46-C4bpa is a potent inhibitor of MV binding to CD46. The preincubation of purified MV with sCD46-C4bpa protein resulted in the abolition of the CD46-mediated specific binding to CHO-CD46 cells at a concentration of >470 nM (equivalent to 3,760 nM monovalent CD46) (Fig. 3a). A similar binding inhibition was observed with 5,000 nM monomeric sCD46. Thus, when their respective valences are taken into account, both proteins have a similar inhibitory binding efficiency. However, while the inhibition curve with the monomeric sCD46 protein shows a regular linear relationship between 625 and 5,000 nM, the corresponding inhibition curve observed with the octameric sCD46-C4bpa protein has a steeper slope. In addition, within the 15 to 120 nM range (equivalent to 120 to 960 nM monovalent CD46), a significant enhancement of MV binding to octameric sCD46-C4bpa was observed, possibly reflecting the binding of virus aggregated in solution by the nonsaturating multimeric protein. When the inhibitor was added after MV binding to CHO-CD46 cells, neither enhancement nor inhibition was observed.

sCD46-C4bpa is a potent inhibitor of MV glycoprotein-induced cell-cell fusion. A quantitative fusion assay based on

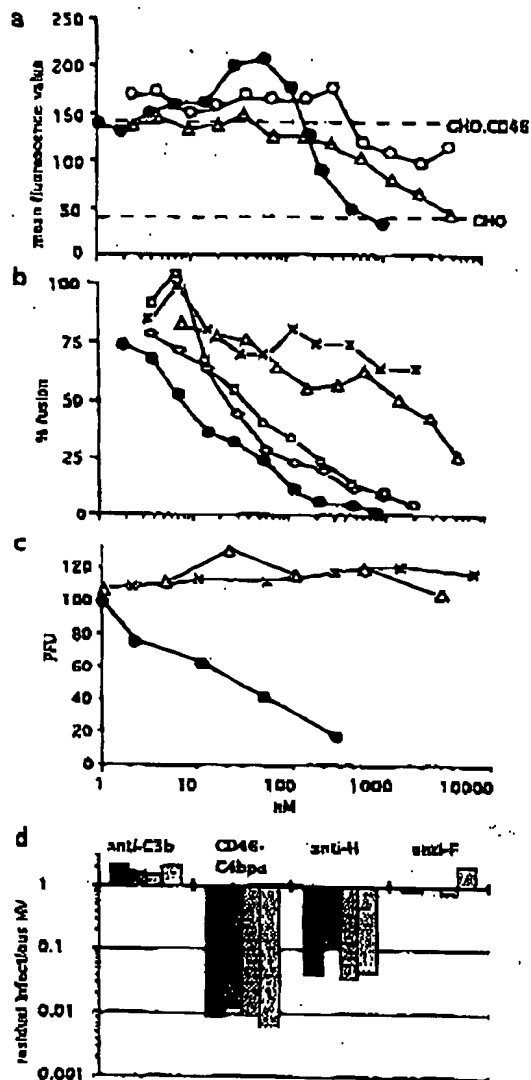


FIG. 3. Inhibition of virus binding (a), virus-induced cell-cell fusion (b), and virus infectivity (c and d). (a) Purified MV was incubated with either sCD46-C4bpa protein (black circles) or sCD46 protein (triangles) before the addition of CHO-CD46 cells; alternatively MV was incubated with CHO-CD46 cells to which the sCD46-C4bpa protein was added afterwards (open circles). (b) Inhibition of fusion in the presence of sCD46-C4bpa protein (circles), sCD46 protein (triangles), 48C16 anti-H (diamonds), Y503 anti-F (squares), or WMI anti-C3b (C1c) (crosses) MAb. The results are expressed as a percentage of the fusion between HeLa and MV-infected HeLa cells observed in the absence of inhibitor as determined by the level of β -Gal activity. (c) MV (100 PFU) was incubated with sCD46-C4bpa protein (circles), sCD46 protein (triangles), or bovine serum albumin (crosses) prior to infection of Vero cells. (d) MV (10^5 , 10^4 , 10^3 , and 10^2 TCID₅₀ [black to light gray columns, respectively]) was incubated with the indicated reagent, and the remaining virus was titrated using the TCID₅₀ assay. The results are expressed as the MV fraction not neutralized. Note that no infectious MV was recovered from 10^5 TCID₅₀ MV incubated with sCD46-C4bpa (i.e., recovery fraction = 0.100).

the conditional expression of β -Gal was used to assess the functional property of the octameric sCD46-C4bpa protein to inhibit cell-cell fusion. After 30 min of preincubation of MV-infected HeLa cells with this protein at 4°C, an almost linear

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decrease in fusion with HeLa cells was observed, with 50% inhibition at 7 nM (equivalent to 56 nM monovalent CD46) and complete inhibition at 950 nM (equivalent to 7,600 nM monovalent CD46). Similar data was also observed following preincubation with anti-MV H and anti-MV F antibodies, although these antibodies were not as efficient, with 50% inhibition being observed at 15 and 22 nM, respectively (Fig. 3b). In comparison, the monomeric sCD46 protein was a poor fusion inhibitor, with 50% inhibitory activity at 1,100 nM (Fig. 3b). As expected the unrelated WM1 antibody had a minimal level of inhibition even at the highest concentration tested. Similar results were obtained when the hamster CHO cells coinfecting with recombinant vaccinia virus coding for MV H and F proteins and CHO-CD46 were used as cell fusion partners. As a control for specificity, every fusion inhibitor was found not to inhibit the cell-cell fusion assay mediated by the closely related CDV.

sCD46-C4bpa is a potent inhibitor of MV infection *in vitro*. When the octameric sCD46-C4bpa protein was incubated with 100 PFU of MV in the presence of Vero indicator cells, it was a potent inhibitor of infection, with 50% inhibitory activity at 17 nM (equivalent to 136 nM monovalent CD46) (Fig. 3c), i.e., consistent with the 50% inhibitory activity observed with cell-cell fusion (7 nM). In contrast, and as previously reported (16, 45), the monomeric sCD46 was unable to neutralize the virus, as was the control bovine serum albumin. To test the reversibility of this neutralizing effect, 10^3 , 10^2 , 10^1 , and 10^0 TCID₅₀ of MV were incubated with a 300-μg/ml concentration (i.e., 950 nM for sCD46-C4bpa) of inhibitor for 1 h and then diluted 100-fold and more (i.e., below the 50% inhibitory activity level observed when sCD46-C4bpa is left throughout the MV neutralization assay (Fig. 3c)). A constant proportion of approximately 99% of MV (i.e., 2 log units) was irreversibly neutralized by the sCD46-C4bpa protein (Fig. 3d). The 48c16 anti-H MAb was also very efficient at neutralizing MV although not as efficient as sCD46-C4bpa, with between 90 and 95% (i.e., 1-log range) of MV being neutralized. Interestingly, despite being a potent inhibitor of MV-induced cell-cell fusion, the Y503 anti-F MAb was unable to irreversibly neutralize the virus. As a control, incubation of MV with WM1 antibody had no effect.

sCD46-C4bpa is a potent inhibitor of MV infection *in vivo*. The neutralizing properties of CD46 reagents were then tested in a transgenic CD46 mouse characterized by (i) high susceptibility to MV infection and productive replication in the brain after intracranial inoculation and (ii) obligatory use of the cellular receptor CD46 by MV (18, 25). When 11 μg (i.e., 35 pmol [equivalent to 280 pmol of monovalent CD46]) of octameric sCD46-C4bpa protein was coinjected intracranially into newborn transgenic CD46 mice with 6,000 PFU of MV (Edmonston strain), all animals survived, whereas mice inoculated with MV alone were all killed, with a mean survival time of 7.6 days (Table 2). In the group of mice inoculated with MV and 24 μg (i.e., 400 pmol) of monomeric sCD46, three out of four mice died, with a mean survival time of 13 days. The protective effect of both octameric sCD46-C4bpa and monomeric sCD46 were specific to MV, since they did not prevent or delay the death induced by the inoculation of transgenic CD46 mice with CDV, which does not use CD46 as a receptor.

DISCUSSION

The fusion of the C4bpa bundle domain to the ectodomain of CD46 resulted in the generation of a chimeric disulfide-bound homo-octameric protein, sCD46-C4bpa. This structure is similar to the homo-octameric C4bp α chains synthesized in the absence of the C4bp β chain (23) and to the homo-oc-

TABLE 2. *In vivo* neutralizing activity of octameric sCD46-C4bpa and monomeric sCD46 proteins^a

Inoculated virus	Treatment	Death ratio ^b	Mean survival time (days)
MV	PBS	6/6	7.6
	sCD46	3/4	13.0
	sCD46-C4bpa	0/6	—
CDV	PBS	3/3	7.3
	sCD46	3/3	7.6
	sCD46-C4bpa	3/3	7.7

^a Suckling transgenic CD46 mice (2 to 3 days old) were inoculated intracranially with either 11 μg (i.e., 35 pmol) of octameric sCD46-C4bpa (equivalent to 280 pmol of monovalent CD46) or 24 μg (i.e., 400 pmol) of monomeric sCD46 or PBS together with 6,000 infectious units of either MV or CDV. Animals were observed for clinical symptoms and death daily during 10 weeks.

^b Number of mice that died/number of mice inoculated.

—, all mice survived.

tameric chimeric anti-Rh(D) Fv antibody (29). Compared to the natural transmembrane CD46 molecule, the octameric sCD46-C4bpa shows a reduced reactivity with two antibodies, anti-SCR II M75 and anti-SCR III and IV GB24, which have a strong inhibitory activity against the CD46 cofactor activity (1, 44) (Table 1). Accordingly, it lacks any cofactor activity, whereas the monomeric sCD46, which shows a reduced reactivity towards only one antibody (GB24) still exhibits a significant cofactor activity. Noteworthy, the SCR II domain of CD46 does not contain primary binding sites for C3b but is required for the cofactor activity, and the SCR III and IV domains contain the binding site for C3b (1). The lack of cofactor activity of sCD46-C4bpa on C3b deposition following alternative complement activation was surprising because the natural C4bp α chain is structurally and functionally related to CD46. Indeed, the C4bp molecule (seven α chains plus one β chain) is a cofactor of factor I for the cleavage of C4b but not of C3b (24, 43). This activity has been mapped to the first three N-terminal domains of the C4bp α chain (24). A monomeric membrane-anchored C4bp α chain protein displays an additional cofactor activity for the factor I-mediated cleavage of C3b, which maps to both N-terminal and C-terminal SCR domains (35). We suggest that the localization of the CD46 SCR III and IV domains adjacent to the bundle region of C4bp α chain effectively hampers binding to C3b and/or cofactor activity through steric hindrance. We do not have a satisfactory explanation for the unexpected enhancement of C3b deposition at intermediate concentrations of both sCD46 and sCD46-C4bpa, although one can speculate about a transient stabilizing effect on the C3bBb convertase, as previously observed with solubilized transmembrane CD46 in the absence of factor I (42).

The SCR I and II domains of sCD46-C4bpa are accessible for binding to the MV H protein. The 2.5-fold-higher avidity of the chimeric protein, compared to that of monomeric sCD46, could be related to cooperative binding of each of the CD46-C4bpa monomers and/or to subtle conformational changes in the H binding site induced by modified interactions with the underlying SCR III and/or IV domains (9, 14). The sCD46-C4bpa protein exhibits a lower reactivity with three anti-CD46 antibodies able to compete with MV interaction, including M75, a very strong inhibitor of the binding of an MV-soluble H (6) (Table 1). This suggests that the local structure of the sCD46-C4bpa protein subtly differs from that of the natural transmembrane CD46 but can still accommodate efficient interaction with MV. This is in agreement with the relative in-

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sensitivity of CD46 to point mutations of amino acids in SCR I and II domains (6, 26, 32). How does the oligomeric structure of the sCD46-C46pc compare with that of natural transmembrane CD46? From cross-linking experiments, CD46 seems to exist as dimers and possibly trimers (31), and the crystal structure of a CD46 SCR I-SCR II fragment revealed a trimeric arrangement (8).

The octamerization of the CD46 ectodomain resulted in a chimeric protein with an anti-MV activity improved by 2 orders of magnitude, thus far exceeding the modest increase of its avidity for the MV H protein. The mechanism of this antiviral activity could be a competition for binding to the cell surface CD46 receptor and/or an irreversible conformational change of the fusion protein induced by the simultaneous binding of several adjacent H companion molecules to the octameric receptor. In favor of the latter, (i) the octameric and monomeric receptors displayed similar efficiencies in saturating CD46 binding sites at equal valency, (ii) at intermediate concentrations, the octameric protein resulted in an increased amount of virus binding and/or uptake with decreased infectivity (compare Fig. 3a and c), and (iii) unlike the anti-F MAb, its neutralizing ability was irreversible in vitro. Moreover, this would explain the potent neutralizing activity of the octameric soluble receptor in vivo.

sCD46-C46pc is derived from two human proteins, is devoid of complement regulatory properties, has a high MM which should increase its serum half-life, and displays potent in vitro and in vivo neutralizing properties. Consequently, it is a good candidate for clinical use in the control of MV infection in immunocompromised patients (2, 4, 36), in patients suffering from acute or subacute encephalitis, and in young children infected at the critical transition age between maternally transmitted antibody protection and a successful anti-measles vaccination coverage (41). Further studies using animals which more closely model the human disease are in progress to validate this new therapeutic concept. The C46pc-based octamerization procedure of a cellular receptor might also prove useful in generating other efficient antiviral reagents.

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6

A Recombinant Human scFv Anti-Rh(D) Antibody With Multiple Valences Using a C-Terminal Fragment of C4-Binding Protein

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Monomeric recombinant molecules prove generally unsatisfactory for *in vivo* use. Most biological systems are indeed multivalent either structurally, associating different chains, or functionally, when cross-linked by their ligands. Mimicking natural molecules for immune intervention implies the need for multimerizing systems to create multivalent molecules capable of interfering with physiological processing. A multivalent anti-Rh(D) recombinant protein has been designed by reconstructing the antibody binding site of a human monoclonal anti-Rh(D) antibody as a single chain Fv mini antibody, then multimerizing it by inserting at its C-

terminal end the C-terminal part of the C4 binding protein (C4bp) alpha chain, which is responsible for the octamer multimerization of that molecule. This soluble multivalent recombinant molecule was functional, bound red blood cells (RBCs), agglutinated them, and did not activate complement. This demonstration model opens the way for future *in vivo* use of multivalent molecules associating antibody valences and other functional molecules for cell targeting, imaging, or removal of cells such as Rh(D)-positive RBCs for preventing Rh alloimmunization.

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MULTIVALENT EXPRESSION of antibody combining sites allows the creation of complexes that are multivalent and can be multispecific and are, thus, susceptible to a spectrum of therapeutic and diagnostic applications.

Multimerization of chimera molecules can be conceived according to different principles. Two possible orientations are direct coupling of the entities concerned or the use of an intermediary multimerizing system through which two or more molecular species can be associated to form heteromultimers. This second approach, susceptible to more general applications in the one we have chosen for the present work. In a therapeutic perspective, to be nonimmunogenic and as inert *in vivo* as possible, the fragment conferring multimerizing potential should derive from a physiological constituent of human plasma and should not activate complement.

The C4 binding protein (C4bp) molecule, a normal plasma protein,^{1,2} is a spider-like structure (570 kD) made of 7 α -chains and 1 β -chain. Binding sites for C4bp molecules are located on α -chains, whereas the protein-S binding site is located on the β -chain. A minor form made of only 7 α -chains is also present at a lower concentration in normal human plasma. A third 5 α /1 β -chain molecule has also been described. The basic repetitive structure of both chains is termed short consensus repeat (SCR). Each SCR of about 60 amino acids includes two intra-chain disulfide bridges.

A minimal C4bp α -C-terminal fragment lacking biological functions has been used to produce a soluble multimeric multivalent single chain Fv (scFv) anti-Rh(D) molecule capable of spontaneous multimerization through the associated

C4bp fragment. These molecules are homomultimers, which maintain their capacity to recognize antigen but have a higher valence number.

We have elected to use an anti-Rh(D) human single-chain Fv as a model system for multimerization. Rh antigen D is borne by a transmembrane unglycosylated protein. Rh antigens, such as Rh(D), can be conceived as an anchoring point for heterochimeric molecules with novel properties such as enzymatic activities. The Rh(D) antigen is responsible for anti-Rh(D) alloimmunization by transfusion and, through materno-fetal immunization, is the most common cause of hemolytic disease of the newborn. Postpartum prevention of alloimmunization is at the present time affected by injection of human polyclonal anti-Rh(D). In the search for a future replacement a multimeric structure of a monoclonal derived anti-Rh(D) scFv is a first step in the construction of a heteromultimeric molecule that can serve to target Rh(D)-positive red blood cells (RBCs) to selected antibody-dependent cell-mediated cytotoxicity effector cells.

MATERIALS AND METHODS

Cloning of Anti-Rh(D) Heavy and Light Chain-Variable Region Coding Sequences and Assembly Into an scFv

A lymphoblastoid cell line, H2DS2F5, derived from the peripheral blood lymphocytes of a hyperimmunized donor,³ and producing a human monoclonal IgG1, λ specific for erythrocyte Rh(D) antigen, was used as a source of monoclonal cells to rescue variable heavy (VH) and light (VL) regions via polymerase chain reaction (PCR) amplification.

The single-chain Fv was constructed according to the method described by Marks et al.⁴ Amplification of VH and VL coding fragments was effected with the specific V gene family primers.⁵ Paired VH and VL were assembled by PCR with a (Gly-Ser)₃ linker, and the assembled structures were cloned into pHEM-1 vector⁶ (kindly provided by Prof G. Winter, M.R.C. Laboratory of Molecular Biology, Cambridge, UK). Clones were tested for expression of soluble scFv after isopropylthiogalactoside induction.⁷ The scFv construct from a clone with high specific reactivity to anti-Rh(D) erythrocytes was selected for construction of the chimeric protein.

Primers Used to Amplify the C-Terminal Part of the C4bp Molecule

The 174-base pair C-terminal C4bp α fragment was amplified using the following primers: 5' primer with MYC Tag, 5'-AGTGGC-

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A RECOMBINANT MULTIMERIC ANTI-Rh(D) scFv

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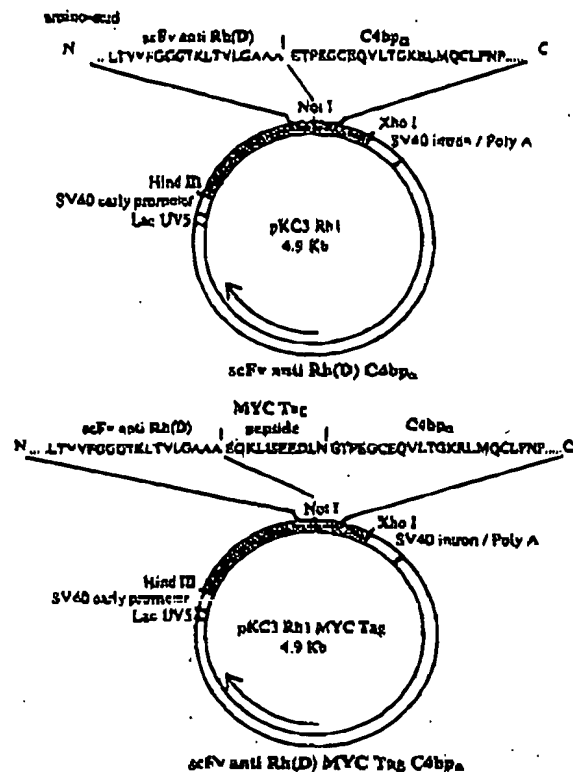


Fig 1. Maps of pKC3 Rh1 and pKC3 Rh1 MYC Tag plasmids coding for multimeric scFv Rh(D) and MYC Tag multimeric scFv Rh(D) respectively. Amino acid sequences of the junction areas between scFv Rh(D) and C-terminal part of C4bp α -chain are depicted above the overall plasmid.

CGCGGAGAA CAAAACTCATCTCAGAAAGAGGATCTG-
AATGAGACCCCGAAGGCTGTGA-3'; 5' primer without MYC
Tag, 5'-AGTGGCGGCGGAGAGACCCCGAAGGCTGTGA-3';
3' primer, 5'-CTCGGCGGCGGCTCGAGTTATAGTTCTTTA-
TCCAAAGTGG-3'.

Underlined sequences represent restriction endonuclease sites. The 5' and 3' primers contain *Not*I and *Xho*I sites, respectively. The sequence depicted in bold characters codes for the MYC Tag peptide.¹⁰ Two different sequences were amplified. The first sequence contained a stop codon and a restriction site for *Xho*I at its 3' end as well as a restriction site for *Not*I at its 5' end. The second sequence also contained these flanking sequences as well as a coding sequence for the MYC Tag peptide at its 5' end, downstream to the *Not*I site. This peptide allowed detection and characterization of the recombinant protein (Fig 1).

PCR Amplification

Genomic DNA was used as a template for PCR amplification. The reaction mixture was subjected to 30 cycles of amplification using a Gene Amp PCR System 9600 (Perkin-Elmer, Foster City, CA). Cycles were of 1 minute at 94°C, 1 minute at 56°C, and 2 minutes at 72°C. DNA was then digested with *Not*I and *Xho*I.

Cloning of the scFv Anti-Rh(D) C4bp Construct (mRh1)

Restriction enzymes and alkaline phosphatase used for cloning were purchased from Boehringer Mannheim (Meylan, France). The T4 DNA ligation kit used was from Ozyme (Montigny-Le Bretonneux, France). The scFv construct was excised from the pHEX-1 vector by digestion at the *Hind*III and *Not*I sites, thus including the bacterial leader peptide *pelB*.¹¹ The amplified C4bp fragment was digested by *Not*I and *Xho*I. These two DNA fragments were linked to a dephosphorylated pKC3 vector¹² digested by *Hind*III and *Xho*I (Fig 1). *Escherichia coli* DH5 α host strain was transformed using the pKC3 scFv anti-Rh(D) C4bp construct (mRh1).

Transfection of Chinese Hamster Ovary (CHO) Eukaryotic Cell Line and Amplification of the Transfected mRh1 Genes

Dihydrofolate reductase (dhfr) negative CHO cells (ATCC CRL-9095; American Type Culture Collection, Rockville, MD) were used for transfection.

Transfection was performed using a calcium phosphate transfection kit (5 Prime-3 Prime Inc, TEBU, Le Perray En Yvelines, France). Plasmid pKC3 scFv anti-Rh(D) C4bp or pKC3 scFv anti-Rh(D) C4bp MYC Tag (Fig 1) was cotransfected with the dhfr selective plasmid ST4.¹³ CHO transformed with the appropriate vectors were selected according to their ability to grow in nucleoside-free medium. The screening of positive clones was performed by direct hemagglutination. Subsequent selective cycling in the presence of increasing concentrations (0.02 to 80 μ mol/L) of amethopterin (Methotrexate; Sigma, St Louis, MO), a potent inhibitor of dhfr function, resulted in an amplification of the integrated DNA and an increased expression of the multimeric scFv.

Direct Hemagglutination

Gel-test and columns were purchased from Institut J. Boy (Reims, France). Twenty microliters of a 2.5% suspension of papain-treated erythrocytes (E) were incubated 30 minutes at 37°C with 50 μ L of pure or diluted supernatants of transfected cells. Agglutination was then assessed in Sepharose columns after a 1,000g centrifugation of 10 minutes at room temperature.

Immunoprecipitation

Anti-MYC Tag monoclonal antibody (MoAb)¹⁴ from MYC 1-9B10.2 (ATCC CRL-1729) cell line was purified using the octanoic acid, contra precipitation method¹⁵ and biotinylated using a hydroxy-succinimide LC biotin linker (Pierce; Interchim, Moulignon, France) according to the manufacturer's instructions.

A total of 3×10^6 cells in a 75-cm² flask were labeled during 24 hours at 37°C using 170 μ Ci/mL ³⁵S methionine-cysteine (Amersham, Les Ulis, France) in 7 mL of methionine and cysteine-free RPMI 1640 medium (ICN, Orsay, France) supplemented with 10% heat-inactivated fetal calf serum and 2 mmol/L glutamine. Magnetic beads coated with sheep anti-mouse IgG (DynaBeads; Dynal, Compiegne, France) were incubated with radiolabeled supernatant. After washing, immunoprecipitated proteins were analyzed in sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) under reducing and nonreducing conditions.¹⁶

Immunofluorescence Assays: Assessment of the Fixation of mRh1 on RBCs

Twenty microliters of a 2.5% suspension of papain-treated Rh(D) positive RBCs was incubated for 45 minutes at 37°C with 50 μ L of mRh1 supernatant, washed twice in phosphate-buffered saline (PBS)

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supplemented with 1% bovine serum albumin, then incubated with 2 μ g of biotinylated anti-MYC Tag MoAb. RBCs were then washed twice and stained with a biotin-avidin-enhancing system, as previously described.¹¹ Stained RBCs were fixed in 0.37% formaldehyde diluted in washing buffer and analyzed on a FACStar Plus (Becton Dickinson, Mountain View, CA). Papain-treated Rh(D)-negative RBCs were used as a negative control. A competition assay using the anti-Rh(D) DF5 human MoAb, from which the scFv originated, was performed to assess the specificity of binding of mRh1 to RBCs. For longitudinal testing experiments, fluorescent calibration beads (Becton Dickinson) and mouse IgG-coated beads (Qifkit; Biotec, Marseille, France) were used to standardize day-to-day settings of the apparatus.

Complement Fixation Tests

Assessment of C activation by mRh1 using hemolytic assay. Fifty microliters of 2.5% suspension of papain-treated Rh(D)-positive RBCs were sensitized by mRh1 at 37°C for 30 minutes in 0.24 mol/L glycine, 3 mmol/L sodium phosphate (pH 6.8), 31 mmol/L NaCl, low ionic strength saline buffer including 0.15 mmol/L Ca^{2+} , and 0.5 mmol/L Mg^{2+} .

Fifty microliters of guinea pig serum were then added (Biomerieux, Marcy l'Etoile, France). Culture medium or MoAb against glycophorin A¹⁴ were used as controls. After 30 minutes incubation at 37°C and a quick cooling in an ice-water bath, tubes were centrifuged for 10 minutes at 700g, then the 403-nm optical density of the supernatants was determined using a microplate reader (SLT, Labinstrument, Visoche, St Bonnet De Mure, France). Complement lysis tests were also performed with an additional step of 30 minutes incubation of 1.5 μ g of anti-MYC Tag MoAb.

Twenty microliters of rabbit antiserum to whole IgG (Cappel, Flbio, Combevoie, France) were then added for another 30 minutes incubation before adding guinea pig serum. Correct attachment of mRh1 on RBCs was checked by flow cytometry analysis following fluorescein isothiocyanate protein A labeling (Sigma).

Assessment of C activation by mRh1 using flow cytometry analysis. Papain-treated Rh(D)-positive RBCs were sensitized by using mRh1, anti-MYC Tag MoAb, and an affinity purified rabbit antibody against mouse IgG and IgM (heavy and light chain) Ig (Pierce). Human C5-deficient serum, twofold diluted in low ionic strength saline including 0.15 mmol/L Ca^{2+} and 0.5 mmol/L Mg^{2+} , was then added, and the mixture was incubated for 30 minutes at 37°C. Human C3b deposits were revealed by fluorescein-labeled goat antiserum against human C3b (Cappel). RBCs were washed twice, stabilized in 0.37% formaldehyde PBS buffer, then analyzed on a FACStar Plus (Becton Dickinson).

RESULTS

Multivalent anti-Rh(D) recombinant proteins were obtained with C4bp and C4bp MYC Tag multimerizing fragments. They were soluble and stable in culture supernatant.

These mRh1 directly agglutinated papain-treated Rh(D)-positive RBCs, as did anti-Rh(D) MoAb DF5 (Fig 2), unlike monomeric anti-Rh(D) scFv (data not shown), and could also, less intensively, agglutinate native RBCs, which the parent DF5 MoAb did not. These recombinant multimers proved stable: Supernatants produced in the course of this work are functional by direct agglutination after 2 years at 4°C, and testing of mRh1 supernatants every week for accelerated aging during 1 month at 37°C showed no significant decrease in staining intensity in flow cytometry binding assays.

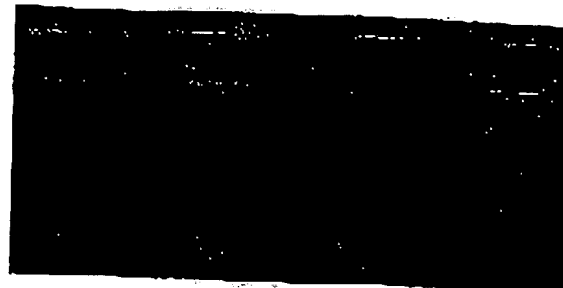


Fig 2. Direct hemagglutination of Rh(D)-positive RBCs by mRh1. (A) Incubation of papain-treated Rh(D)-positive RBCs with DF5 MoAb (positive control). (B) Incubation of papain-treated Rh(D)-positive RBCs with mRh1. (C) Incubation of papain-treated Rh(D)-negative RBCs with mRh1. (D) Incubation of papain-treated Rh(D)-negative RBCs with culture medium (negative control). Agglutinated RBCs remained at the top of the columns.

A first experiment of biosynthetic ³⁵S labeling followed by immunoprecipitation on a bulk of mRh1-transfected cells led to a pattern made of three bands in the range of 200 to 300 kD, the higher band being largely predominant. This pattern was compatible with a mixture of 6-, 7-, and 8-valence multimers, a counterpart of the physiological pattern of C4bp multimers that consists mainly of octamers together with a few heptamers and hexamers. After cloning by limiting dilution, a clone only secreting the molecule of the highest molecular weight was chosen and subsequently developed. ³⁵S amino-acid labeling experiments showed that a single molecular species of 270 kD apparent molecular weight on SDS-PAGE analysis was secreted by this mRh1-transfected CHO cell clone. The molecular weight of its monomeric component was assessed from SDS-PAGE under reducing conditions and was found to be 27 kD. These data suggested that the molecular formula of the multivalent molecule mRh1 was an octamer, like the natural major C4bp component (Fig 3).

The scFv MYC Tag product did not lend itself well to quantification, possibly because of the position of the tag at the center of the spider-like structure. With the same vector, other multimerized molecules (CD4 or CD16) regularly reach a stable secretion level of 1 to 3 μ g/mL, which can be assumed as the secretion range of mRh1, whereas, with a more powerful vector, engineered CD46 reaches a 10 to 20 μ g/mL secretion level (manuscript in preparation).

Flow cytometry analysis showed that the binding of mRh1 was strikingly better on papain-treated RBCs and during a warm incubation.

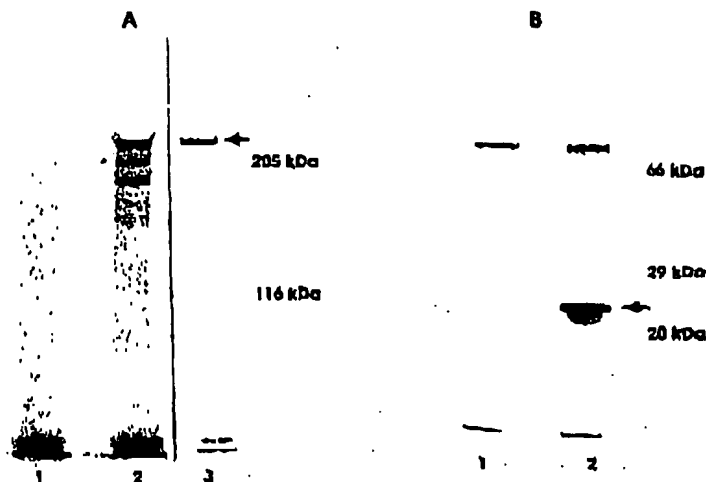
Flow cytometry competition experiments between mRh1 and human anti-Rh(D) DF5 MoAb, from which originated the binding site of mRh1, showed a dose-dependent inhibition validating the specificity of mRh1 binding on RBCs. In complement lysis experiments, no complement fixation was found on mRh1 coated RBCs whereas a significant lysis was observed when an anti-MYC Tag and an antimouse Ig dual

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Fig 3. SDS-PAGE analysis of ³⁵S-labeled supernatants immunoprecipitated using anti-MYC Tag MoAb. Immunoprecipitates were run on 5% (A) and 10% (B) polyacrylamide gels under nonreducing (A-2 and A-3) and reducing conditions (B-2). Untransfected CHO cell immunoprecipitates served as negative controls (A-1 and B-1). Lane A-2 depicts a three-band heterogeneous hexamer, heptamer, and octamer profile observed under nonreducing conditions in a biosynthetic labeling experiment from a bulk of transfected cells before final cloning of cells.



antibody enhancing system was used as a positive control of both mRhl binding and complement potency (Fig 4).

Taken together, these data showed the secretion by mRhl-transfected CHO cells of a unique soluble multimeric molecule with multivalent antibody properties, devoid of any complement fixation activity.

DISCUSSION

The use of molecules of human origin for therapy began with the use of polyclonal whole antisera. MoAb allowed production of better defined molecules, which led to reproducible and well-controlled products. However, their use remained mostly limited to situations in which the destruction of a target is needed because of the properties of the Fc fragment of Igs. Artificial structures such as single chain antibodies^{1,2,3,4,5} generally have a short half-life, have a low avidity because of their single valence, and are, in most circumstances, unable to trigger biological functions alone.

The association of different structures in a given recombinant molecule has been hampered by problems because of conformation and accessibility. Different approaches have been proposed to obtain bivalent or multivalent molecules. Chemical linkage of proteins to polyethylene-glycol or dextran is cumbersome, requiring large amounts of purified material.²² Disulfide linking of Fcy or use of Fcy fragments to create multivalent Ig molecules^{1,2,3,4} has been proposed to maintain and amplify Fc-mediated functions. However the enhancement of Fc-associated reactivity, such as antigen-independent complement activation, can be undesirable in a therapeutic context. The molecules synthesized in these systems have been heterogeneous, varying from monomers to hexamers and higher order structures. Dimerization of smaller F(ab')₂ or Fv structures has been effected through the use of amphipathic helices, either leucine zippers or bundle-helix constructs.^{20,21} The former do not satisfactorily react to the introduction of a covalent link and, thus, are potentially unstable; furthermore, these molecules are not natural

components of plasma and may prove immunogenic. Other approaches to dimerization of scFv have been proposed,²² such as diabodies.²³ Nevertheless dimeric structures may not be optimal for given applications.

Multimeric constructions, such as those described by Ito et al.²⁴ based on fusion to protein A, can generate complexes of Fv-protein A and IgG with variable stoichiometry or by Dübel et al.²⁵ in which scFv are fused to a core-streptavidin structure allowed the creation of tetrameric antibodies with additional coupling possibilities because of the presence of biotin binding sites and cysteines. The use of a heteroantigenic fragment, protein A, or streptavidin carries the risk of an immune response which could seriously limit potential therapeutic applications.

The C-terminal C4bp multimerizing system fulfills the requirement of a multi-purpose multimerizing system for future in vivo use.

This study established that the C terminal part of the α -chain of C4bp is sufficient to induce polymerization during protein synthesis, and, although the expression vector codes only for monomers, multimers are assembled in the cell without necessity for secondary modifications resulting in the secretion of a unique, covalently linked soluble molecule. This research model can be further optimized to meet the needs of high-level production. Clearly, production system, vectors, leader sequence and junction area at the 5' end of the C4bp multimerizing system have to be optimized for that purpose. The multimerizing structure originates from a normal component of human plasma avoiding immunization. It does not impair the solubility of multimeric molecules and lacks any biological function. Although only electron microscopy images will resolve the question with certainty, it seems reasonable to believe that the three multimeric forms observed before cloning were the counterpart of the physiological forms of C4bp, because their relative proportions were also in accordance with the physiological pattern. There was a major representation of structures with eight valences.

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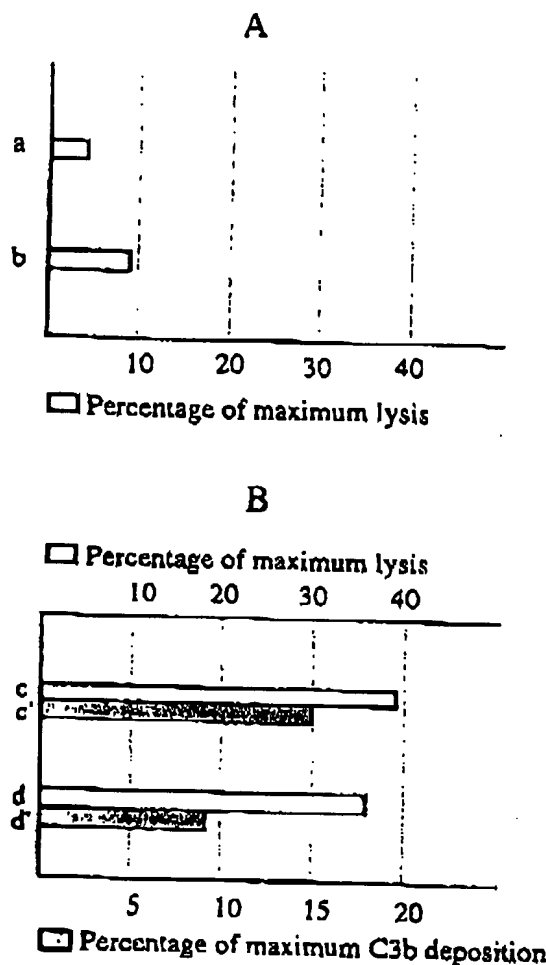


Fig 4. Lack of activation of the complement system by the mRh1. (A) Lack of complement-dependent lysis of RBCs sensitized by using mRh1. (a) mRh1-sensitized RBCs. (b) Control RBCs. (B) Positive control. (c) RBCs sensitized using mRh1 and an anti-MYC Tag and anti-mouse Ig enhancing system. (d) Control RBCs lacking mRh1 incubation step. (c' and d') Immunofluorescence controls of mRh1 and enhancing system fixation. Scale is expressed in percentage as a ratio of the sample mean fluorescence channel to that of an anti-glycophorin A MoAb positive control. Hemolysis is quantified as percentage of the maximal lysis of the system when using a complement activating anti-glycophorin A MoAb.

a higher number than reached by any other multimerizing system. Although no definite explanation is available for the variations in the structure of multimers produced by different cell clones, they could be prone to variation in protein synthesis rate and/or to interference of other protein syntheses in the polymerization process. A possible effect of sequence variation appears less likely, because no clone only secreting either hexamers or heptamers without octamers has been detected, but this hypothesis cannot be totally ruled out. The

multimerizing system provided the multimeric mRh1 with the ability to agglutinate RBCs, suggesting an improvement of the scFv binding through its multivalent nature. The lack of complement activation from mRh1-coated RBCs suggested a promising potential of this system for future in vivo harmless targeting onto cells, here considered either as transporters of various biologically active molecules or for localization by imaging of cells expressing an inappropriate antigen.

Multibodies may also be developed from this model of multi-scFv, associating different antibody binding sites against various epitopes of a given antigen, to maintain the avidity of polyclonal antisera by mimicking the cooperative effect of different antibodies in polyclonal antisera.

Future developments of this system towards heterofunctional multimeric molecules will include the use of C terminal parts of both α - and β -C4bp chains or the use of modified α -chains to control the ratio of two components in heterochimeric molecules and to modulate the polymerization process. These heterofunctional multimeric molecules will either retain the lack of complement activation properties of this system, for example for enzyme replacement therapy or imaging, or will incorporate complement activating or cell attracting molecules to promote the clearance of targeted cells. In the future, new recombinant molecules for clearing Rh(D)-positive RBCs and preventing Rh alloimmunization may be designed from this system.

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